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14. ABSTRACT

Our research program is to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject is to understand the function of a novel estrogen receptor variant, ER-α36, in antiestrogen resistance. In the past year, we have accomplished most of the tasks proposed. We demonstrated that antiestrogen treatment enriched breast cancer stem/progenitor cells and antiestrogens positively regulated the self-renewal of breast cancer stem/progenitor cells. ER-negative breast cancer cells with knocked-down levels of ER-α36 have less cancer stem/progenitor cells and are sensitive to antiestrogens. We found that ER-α36 mediates agonist activities of antiestrogens such as activation of the PI3K/AKT signaling pathway. In addition, we discovered a novel anticancer agent Broussoflavonol that is able to down-regulate ER-α36 expression, induce differentiation of ER-negative breast cancer stem cells and inhibit growth of these cells. Our results thus demonstrated that ER-α36 plays an important role in the resistance of breast cancer stem/progenitor cells to antiestrogens and provided a rational to development of novel therapeutic approaches by targeting ER-α36, which will ultimately revolutionize current therapeutic approaches.

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INTRODUCTION

Our research program is to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance of a novel estrogen receptor variant, ER-α36, in resistance of breast cancer stem/progenitor cells to antiestrogens. In the past year, we have accomplished most works proposed in the original grant. As reported in my last year's progress report, we demonstrated that antiestrogen resistant ER-positive breast cancer cells contain high populations of stem/progenitor cells, and the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. We also found that ER-α36 plays an important role in maintenance of ER-positive breast cancer stem/progenitor cells and contributes to the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens through mediating agonist activities of antiestrogens. In the past year, we extended our research into ER-negative breast cancer stem/progenitor cells. We found that ER-α36 is important in maintenance of the stem/progenitor cells from ER-negative breast cancer cells. ER-negative breast cancer cells with knocked-down levels of ER–α36 expression contain less cancer stem/progenitor cells and that ER-negative breast cancer cells with knocked-down levels of ER-α36 were more sensitive to tamoxifen. In ER-negative breast cancer stem cells, antiestrogen reduced spontaneous differentiation.

Further study of the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance will not only provide important information about the function of breast cancer stem/progenitor cells in development of antiestrogen resistance, but will also lay the foundation for development of novel therapeutic approaches to interfere with antiestrogen resistance.

BODY

Task 1: To determine whether the breast cancer stem/progenitor cells from ER-positive breast cancer cells are involved in antiestrogen resistance and the function of ER-α36 in the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens (months 1-16).

In the previous report, we reported our findings that ER-positive breast cancer stem/progenitor cells enriched with the ALDH1 and CD24-/C44+ markers are insensitive to antiestrogens. We also reported that the antiestrogen insensitive HER-expressing cells contain high population of breast cancer stem/progenitor cells. In addition, antiestrogens were able to expand the populations of ER-positive stem/progenitor cells and to increase the self-renewal capabilities of breast cancer stem/progenitor cells. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation.

In the past year, we accomplished most experiments proposed as detailed in the approved Statement of Work.

1e. To establish stable cell lines from MCF/TAM and MCF7/HER-2/18

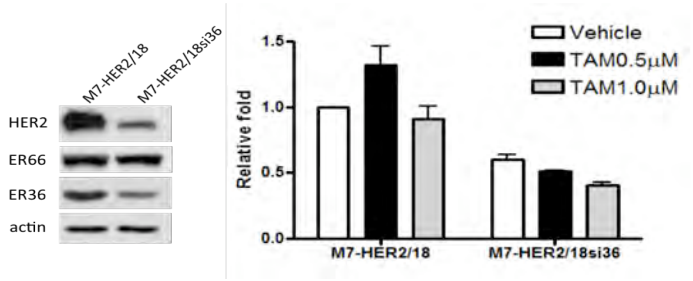

Fig 1. Knock-down of ER-α36 expression sensitized MCF7/HER2/18 breast cancer stem/progenitor cells to tamoxifen.

cells with ER-α36 expression "knocked-down" using the shRNA approach (months 2-6, from SOW).

We have successfully used the Lentivirus-based ER-α36 shRNA system to express ER-α36 shRNA in MCF7/HER-2/18 cells and established a stable cell line from MCF7/HER-2/18 with knocked-down levels of ER-α36 (MCF7/HER-2/18si36 (**Fig. 1**). We also found knockdown of ER-α36 expression sensitized these HER2-expressing breast cancer stem/progenitor cells to tamoxifen (**Fig. 1**).

1h. To perform in vivo assays to assess tumor seeding efficiency of the antiestrogen resistant stem/progenitor cells from ER-positive breast cancer cells; 240 female nude mice will be used (months 10-16, from SOW).

We have finished these experiments as proposed. To assess tumor-seeding efficiency of the ER-positive breast cancer MCF7 and T47D cells survived 1μM of TAM and ICI 182, 780, presumably the stem/progenitor cells. Antiestrogen resistant cells at 1 X 10^2 and 1 X 10^3 were re-suspended in 0.1 ml of Matrigel and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (5-6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory). The mice were also implanted with the placebo or 0.35 mg/60-day slow-release estrogen pellets (Innovative Research) to facilitate tumor formation of these cells. Eight mice for each group were used. ER-positive breast cancer cells survived antiestrogen treatment exhibited potent tumor seeding efficiency; generating tumors at 100 cells while ER-positive breast cancer cells treated with vehicle required 10,000 cells to generate tumors (**Fig. 2 and data not shown**). Our results thus strongly suggested that antiestrogen treatment enriched tumor-initiating cells, i.e. breast cancer stem/progenitor cells.

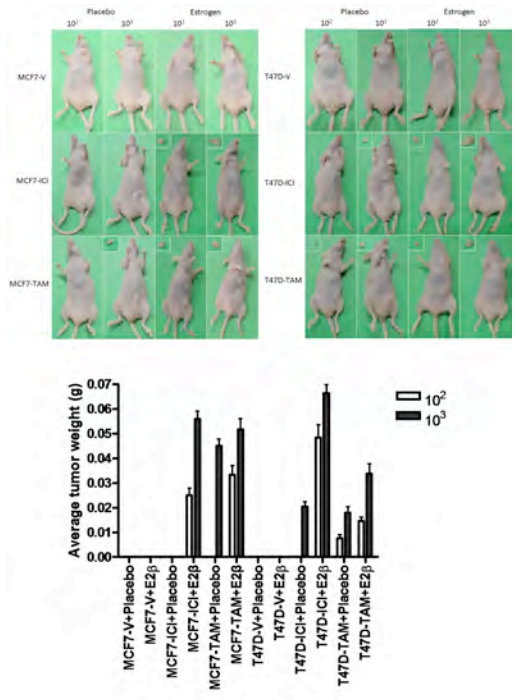

Fig 2. ER-positive breast cancer cells survived antiestrogen treatment exhibited potent tumor-seeding efficiency.

1i. To examine ER-α36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens using cell lines with forced expression and down-regulated expression of ER-α36 (months 8-14, from SOW).

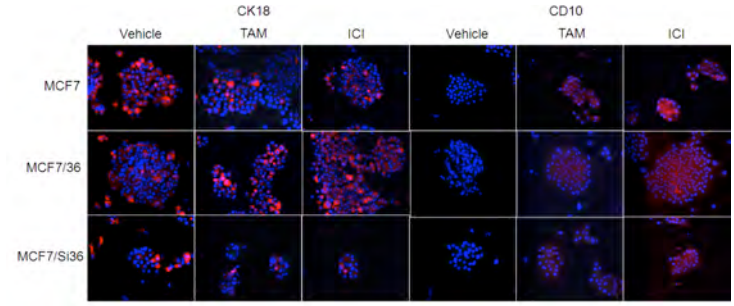

Fig 3. The effects of antiestrogens on the differentiation of ER-positive breast cancer stem cells expressing different levels of ER-α36.

In the previous report, we reported that ER-α36 plays an important role in development of tamoxifen resistance. Elevated ER-α36 expression is one of the mechanisms underlying the development of tamoxifen resistance presumably though mediating

agonist activity of tamoxifen (**See the appendix 1**). We also reported that ER-α36 is involved in positive regulation of breast cancer stem/progenitor cells and high levels of ER-α36 expression contributes to the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens (**See the appendix 1)**.

To investigate the function of ER-α36 in the differentiation lineages of ER-positive breast cancer stem/progenitor cells treated with antiestrogens, tumorspheres formed by ER-positive breast cancer MCF7 and T47D cells with different levels of ER-α36 expression were treated with vehicle or antiestrogens for five days, and examined with indirect immunofluoresces assay to determine differentiation lineages of these cells using cytokeratin 18 for epithelial cells, CD10 for myoepithelial cells. We found that antiestrogen treatment increased the number of cells expressing CK18 or CD 10 in MCF7 cells expressing ER-α36 while failed to do so in the MCF7 cells with knocked-down levels of ER-α36 expression (**Fig. 3**). ICI 182, 780 treatment showed a similar result (**Fig. 3**). ER-positive breast cancer T47D cells behaved the same (**Fig. 3**), suggesting that antiestrogen may induce differentiation of ER-positive breast cancer stem/progenitor cells into both luminal and myoepithelial cell lineages and ER-α36 is involved in this process.

To assess the effects of antiestrogens on the self-renewal of the stem/progenitor cells enriched from ER-positive breast cancer cells with different levels of ER-α36 expression, we studied the tumorsphere formation of MCF7 and T47D cells as well as their derivatives with different levels of ER-α36 expression through serial passage in the absence or presence of estrogen. The cells were treated with vehicle or 1 μM of tamoxifen at the time of each seeding. All viable cells were determined at the end of each passage and seeded for next passage for a total of four passages. We found that the MCF7 and T47D control cells produced more tumorspheres in the 2^{nd}, 3^{rd} and 4^{th} generations in the absence of tamoxifen while tamoxifen treatment further increased the number of tumorspheres in each generation (**data not shown**). Compared to the vector control cells, MCF7/36 and T47D/36 cells generated much more breast cancer stem/progenitor cells in 2^{nd}, 3^{rd} and 4^{th} generations of the self-renewal in the absence of tamoxifen, and tamoxifen treatment further enhanced growth of these cells. In the absence and presence of tamoxifen, MCF7/Si36 and T47D/Si36 failed to generate more tumorspheres in each generation. Our results thus suggested that ER-α36 mediates the agonist activity of tamoxifen in the self-renewal of ER-positive breast cancer stem cells.

The experiments to assess the effects of ICI 182, 780 on the self-renewal of the stem/progenitor cells enriched from ER-positive breast cancer cells with different levels of ER-α36 expression is under way.

Task 2: To investigate the function and the underlying mechanisms of ER-α36 in antiestrogen resistance of the ER-negative breast cancer stem/progenitor cells (months 13-24).

We have accomplished most projects proposed for the task 2.

2a. To determine whether ER-negative breast cancer cells have increased populations of stem/progenitor cells compared to their variants with the knocked-down levels of ER-α36 expression (months 13-14, from SOW).

In the last report, we reported that ER-α36 is important in maintenance of the stem/progenitor cells from ER-negative breast cancer cells SKBR3. To confirm this result, we established stable cells with knocked-down levels of ER-α36 expression in triple-negative breast

cancer MDA-MB-231 and MDA-MB-436 cells **(Fig. 4**). The cells with knocked-down levels of ER-α36 expression also expressed down-regulated levels of EGFR **(Fig. 4**), consistent with our previous report that there is a positive regulatory loop between ER-α36 and EGFR expression that fuels the malignant growth of triple-negative breast cancer (Zhang *et al.*, 2011). The cells with knocked-down levels of ER-α36 expression grew significantly slower compared to the control cells transfected with the empty expression vector. The ER-negative breast cancer cells with knocked-down levels of ER–α36 expression also contain less cancer stem/progenitor cells (**Fig. 5**).

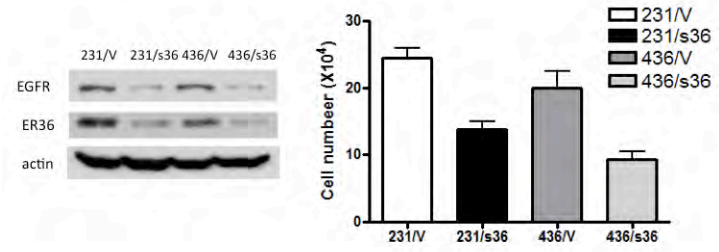

Fig 4. Growth of ER-negative breast cancer MDA-MB-231 and MDA-MB-436 cells with knocked-down levels of ER-α36 expression.

2b. To examine the effects of antiestrogens on the enriched stem/progenitor cells from the ER-negative breast cancer cells with different levels of ER-α36 expression and compare to un-enriched ER-negative breast cancer cells to determine if ER-negative stem/progenitor cells are more resistant to antiestrogens (months 13-16, from SOW).

To examine the effects of tamoxifen on the stem/progenitor cells enriched from the ER-negative breast cancer cells with knocked-down levels of ER-α36 expression, we employed tumorsphere formation assays using MDA-MB-436 and MDA-MB-231 cells transfected with ER-α36 shRNA expression vector. Cells were plated at low density (5,000 viable cells/well) in 6-well ultra-low attachment plates for seven days in the absence and presence of different concentrations of tamoxifen. The tumorspheres formed were dissociated into single cells and the cell number was counted. We found that ER-negative breast cancer cells with knocked-down levels of ER-α36 were more sensitive to tamoxifen (**Fig. 5**). This data thus indicated that down-regulation of ER-α36 sensitizes ER-negative breast cancer cells to tamoxifen.

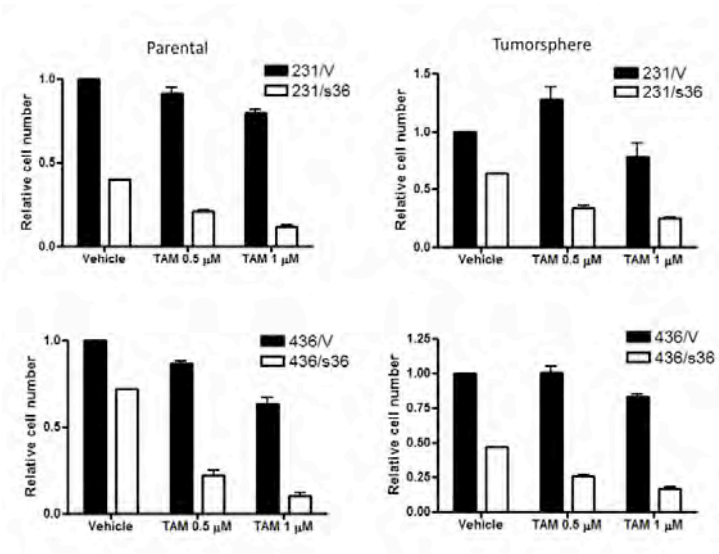

Fig 5. Knock-down of ER-α36 expression sensitizes ER-negative breast cancer MDA-MB-231 and MDA-MB-436 cells to tamoxifen.

We also examined the effects of antiestrogens on differentiation of ER-negative breast cancer stem cells. Tumorspheres formed by ER-positive breast cancer MDA-MB-231 and SKBR3 cells were treated with vehicle, estrogen (E2) or antiestrogens for five days, and examined with indirect immunofluoresces assay to determine differentiation lineages of these cells using cytokeratin 18

(CK18) for epithelial cells, CD10 for myoepithelial cells and vimentin for mesenchymal cells. We found that antiestrogen treatment decreased the number of cells expressing CK18 or CD 10 in SKBR3 cells while failed to influence vimentin expressing cells (**Fig. 6**). ER-negative breast cancer MDA-MB-231 cells showed a similar result (**data not shown**). Our results suggested that antiestrogens may reduce differentiation of ER-negative breast cancer stem cells.

Currently, we are investigating the effects of antiestrogens on the self-renewal and differentiation of the stem/progenitor cells from ER-negative breast cancer cells with knocked-down levels of ER-α36 expression.

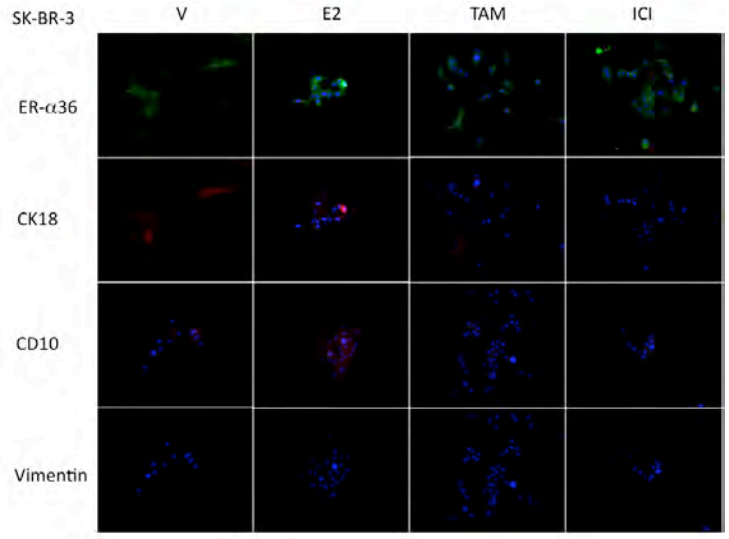

Fig. 6. The effects of antiestrogens on the differentiation of ER-negative breast cancer stem cells from SKBR3 cells.

2c. To determine if ER-α36 can be used as a novel marker for breast cancer stem/progenitor cells (18-24, from SOW).

To examine the possibility to use ER-α36 as a surface marker for breast cancer stem/progenitor cells, we sorted ER-α36 highly expressing cells to examine the stemness of these cells. In the last report, we reported that our polyclonal ER-α36 antibody inhibited growth of sorted cells. In the past year, we established a library of ER-α36 specific monoclonal antibodies and successfully identified an antibody (3C11) that is able to specifically recognize ER-α36 but unable to inhibit cell growth (**Fig. 7**). We are currently characterizing the stem cell features of these ER-α36 positive cells.

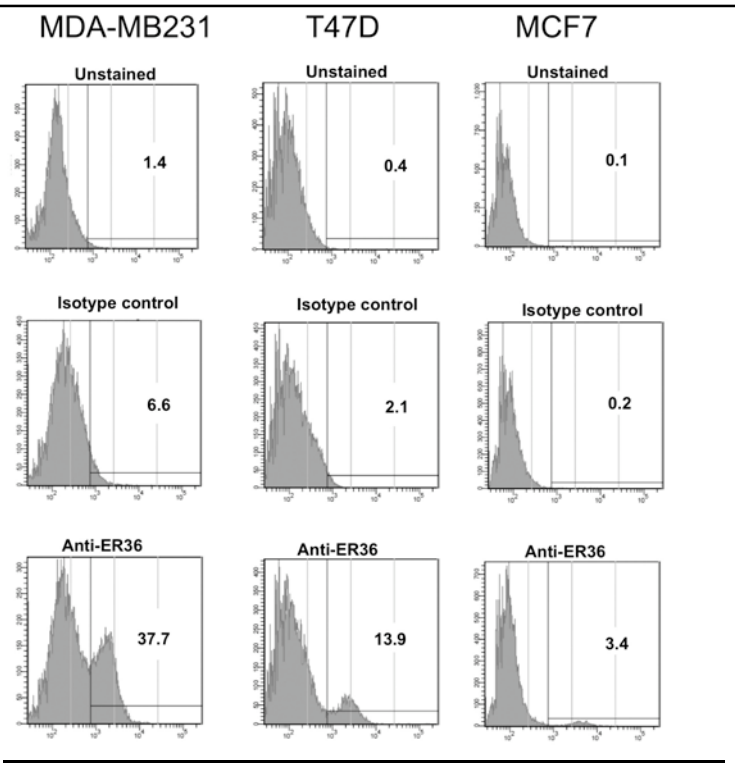

Fig. 7. Flow cytometry sorting of ER-α36 positive cells..

2d. To investigate the underlying mechanisms of ER-α36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens (months 17-24, from SOW).

We recently reported that ER-α36 mediates agonist activities of both TAM and ICI182, 780 in ER-negative breast cancer cells (Zhang *et al.*, 2012) and ER-α36 mediates TAM-induced AKT phosphorylation in TAM resistant MCF7 cells (**see the appendix 1**). In the past year, we

further studied the underlying mechanisms of ER-α36 function in ER-positive breast cancer stem/progenitor cells enriched from MCF7 and T47D cells with knocked-down levels of ER-α36. We found both tamoxifen and ICI 182, 780 activated the PI3K/AKT signaling while failed to do so in the stem/progenitor cells enriched from the cells with knocked-down levels of ER-α36 expression (**Fig. 8**). These results strongly indicated that ER-α36-mediated the PI3K/AKT signaling induced by antiestrogens is one of the mechanisms by which the ER-positive breast cancer stem/progenitor cells that express high levels of ER-α36 become antiestrogen resistant. In the next year of no-cost extension, we will continue our research on the PI3K/AKT signaling pathway in ER-negative breast cancer stem/progenitor cells to examine the possibility that down-regulation of ER-α36 expression or inhibition of the PI3K/AKT pathway will sensitize ER-negative breast cancer stem/progenitor cells to antiestrogens.

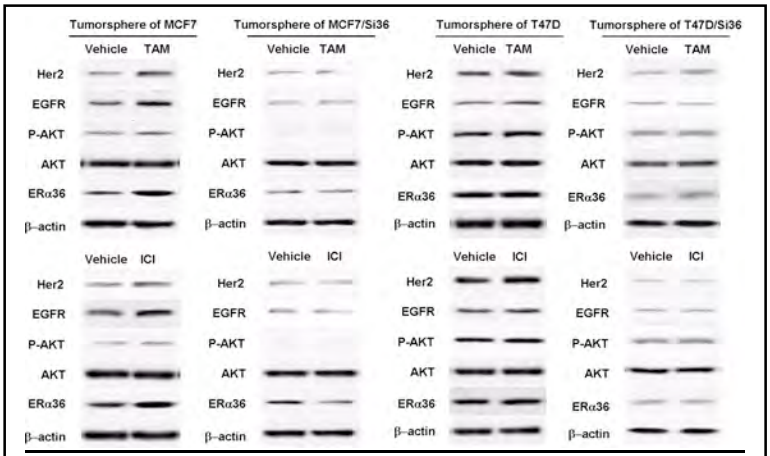

Fig. 8. Activation of the PI3K/AKT signaling by antiestrogens in the cells with different levels of ER-α36 expression.

During our research, novel findings that are consistent with the goal of the grant and that also provide additional support to the hypothesis of the grant have also been made. Based on the importance of ER-α36-mediated signaling in maintenance and regulation of cancer stem/progenitor cells in both ER-positive and –negative breast cancer, we hypothesized that ER-α36 may serve as a target to develop novel therapeutic agents to eradicate breast cancer stem/progenitor cells. Recently, we found that several flavonoid derivatives purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) (L.) were able to down-regulate ER-α36 expression (Guo et al., 2013). We then examined the growth inhibitory activity of the most potent ER-α36 down-regulator Broussoflavonol B in the breast cancer stem/progenitor cells derived from ER-negative breast cancer SK-BR-3 and MDA-MB-231 cells. We found that Broussoflavonol B, as a potent inhibitor of ER-α36 expression, attenuated growth of ER-negative breast cancer stem/progenitor cells and also induced differentiation of these cells (**see the appendix 2 & 3**). These results provided experimental proof for the hypothesis that ER-α36 can serve as a target to develop novel and effective therapeutic approaches to "de-stem" breast cancer stem cells.

KEY RESEARCH ACCOMPLISHMENTS

1. ER-α36 is important in maintenance of ER-negative breast cancer stem/progenitor cells.
2. ER-negative breast cancer stem/progenitor cells with knocked-down levels of ER-α36 are sensitive to tamoxifen.
3. Antiestrogens reduced spontaneous differentiation of ER-negative breast cancer stem cells.
4. ER-α36 can be used as a novel marker for breast cancer stem/progenitor cells.
5. ER-α36-mediated agonist activity of tamoxifen positively regulates the self-renewal of ER-positive breast cancer stem cells.

6. Antiestrogen treatment enriched tumor-initiating cells, i.e. breast cancer stem/progenitor cells.

REPORTABLE OUTCOMES

Zhang, X.T. and Wang, Z-Y. "Estrogen Receptor-α Variant, ER-α36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity". *Endocrinology*. 2013, 154: 1990-1998

Guo, M. X., Wang, M. L., Zhang, X. T., Deng H. and Wang, Z. Y. "Broussoflavonol B Restricts Growth of ER-negative Breast Cancer Stem-like Cells". *AntiCancer Research.* 2013, 1873-1880.

Guo, M. X., Wang, M. L., Zhang, X. T., Deng H. and Wang, Z. Y."A Novel Anticancer Agent Broussoflavonol B Downregulates ER-α36 Expression and Inhibits Growth of ER-negative Breast Cancer MDA-MB-231 Cells". *European Journal of Pharmacology,* 2013, 714: 56-64.

CONCLUSIONS

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen (TAM) and fulvetrant (ICI 182, 780, ICI) provides a successful treatment option for ER-positive breast cancer patients in the past four decades. However, despite the significant anti-neoplastic activity of antiestrogens, most breast tumors are eventually resistant to antiestrogen therapy, which largely affects the efficacy of antiestrogen treatment. The exact mechanisms underlying the antiestrogen resistance in these ER-positive tumors have not been well established.

In this grant, we proposed to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance of a novel estrogen receptor variant, ER-α36, in resistance of breast cancer stem/progenitor cells to antiestrogens. In the past two years, we have accomplished most of the works proposed in the original grant.

In the previous report, we reported that ER-positive breast cancer stem/progenitor cells enriched with the ALDH1 and CD24-/C44+ markers are insensitive to antiestrogens. We also reported that the tamoxifen resistant MCF7 cells and HER-expressing cells contain high population of breast cancer stem/progenitor cells. In addition, antiestrogens were able to expand the population of ER-positive stem/progenitor cells and to influence the self-renewal capabilities of breast cancer stem/progenitor cells. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. These results provided strong evidence to support the hypothesis that breast cancer stem/progenitor cells are involved in antiestrogen resistance, and increased populations of breast cancer stem/progenitor cells is a novel and important mechanism underlying development of antiestrogen resistance.

We also reported that the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells were refractory to antiestrogens TAM and ICI. Antiestrogens TAM and ICI at low concentrations (< 1μM) even stimulated proliferation of the stem/progenitor cells. Now, we report that antiestrogen treatment enriched tumor-initiating cells, i.e. breast cancer stem/progenitor cells.

We concluded that breast cancer stem/progenitor cells are resistant to antiestrogens and antiestrogens influence both proliferation and differentiation of breast cancer stem/progenitor

cells. Thus, antiestrogen treatment enriches breast cancer stem/progenitor cells that are resistant to antiestrogens. This suggests that antiestrogen therapy, while killing the bulk of breast tumor cells, may eventually fail since they do not eradicate breast cancer stem cells that survive to regenerate new tumors.

Now, in this report, we further demonstrate that a novel estrogen receptor variant, ER-α36, plays an important role in positive regulation of both ER-positive and –negative breast cancer stem/progenitor cells and is involved in the resistance of breast cancer stem/progenitor cells to antiestrogens through mediating agonist activities of antiestrogens. We found that ER-α36 mediates the agonist activity of tamoxifen and positively regulates the self-renewal of ER-positive breast cancer stem cells. Antiestrogens also influence differentiation of ER-positive breast cancer stem cells into both luminal and myoepithelial cell lineages and ER-α36 is involved in this process. We also found that knockdown of ER-α36 expression sensitized HER2-expressing breast cancer stem/progenitor cells to tamoxifen. These findings are of both biological and clinical significance.

In the previous report, we reported that ER-α36 is important in maintenance of the stem/progenitor cells from ER-negative breast cancer SKBR3 cells. Now, we found that the ER-negative breast cancer cells with knocked-down levels of ER–α36 expression also contain less cancer stem/progenitor cells and that ER-negative breast cancer cells with knocked-down levels of ER-α36 were sensitive to tamoxifen. This data thus suggested that down-regulation of ER-α36 sensitizes ER-negative breast cancer stem/progenitor cells to antiestrogens. In ER-negative breast cancer stem cells, antiestrogen reduced spontaneous differentiation, suggesting that antiestrogens may maintain the “stemness” of ER-negative breast cancer stem cells.

In the past year, we further explored the underlying mechanisms of ER-α36 function in ER-positive breast cancer stem/progenitor cells enriched from MCF7 and T47D cells with knocked-down levels of ER-α36. We found both tamoxifen and ICI 182, 780 activated the PI3K/AKT signaling while failed to do so in the stem/progenitor cells enriched from the cells with knocked-down levels of ER-α36 expression. These results strongly indicated that ER-α36-mediated PI3K/AKT signaling induced by antiestrogens is one of the mechanisms by which the breast cancer stem/progenitor cells that express high levels of ER-α36 become antiestrogen resistant.

During our research of last year, we also found that a flavonoid derivative Broussoflavonol B, inhibited the expression of ER-α36 and EGFR, attenuated growth of ER-negative breast cancer stem/progenitor cells and also induced differentiation of these cells. These results provided experimental proof for the hypothesis that ER-α36 can serve as a target to develop novel and effective therapeutic approaches to “de-stem” breast cancer stem cells.

The discovery of breast tumor cells that behave like stem cells and that are resistant to chemotherapy drugs, radiation therapy and antiestrogens provided a reasonable explanation for the difficulty to eradicate breast cancer. Novel mechanisms and targets for development of effective therapeutic approaches to inhibit growth of breast cancer stem cells are urgently needed. Our finding that the Broussoflavonol B, as a potent inhibitor of ER-α36 expression, inhibited growth of ER-negative breast cancer stem/progenitor cells provided a rational to development of novel therapeutic approaches by targeting ER-α36, which will ultimately revolutionize current therapeutic approaches.

During the next year of no-cost extension funding, it is planned to finish the task 1 and further pursue the work of task 2 as detailed in my Approved Statement of Work.

Task 1: To determine whether the breast cancer stem/progenitor cells from ER-positive breast cancer cells are involved in antiestrogen resistance and the function of ER-α36 in the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens (months 1-16).

1g. To study the abilities of the stem/progenitor cells enriched from HER2 expressing cells to form tumorspheres, self-renewal and differentiation (months 10-14, from SOW).

We will finish the examination of the effects of different concentrations of tamoxifen and ICI 182,780 on the self-renewal and differentiation of the stem/progenitor cells enriched from HER-2 expressing BT474 and MCF7/Her2/18 cells.

1i. To examine ER-α36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens using cell lines with forced expression and down-regulated expression of ER-α36 (months 8-14, from SOW).

We will finish the experiments to assess the effects of ICI 182, 780 on the self-renewal of the stem/progenitor cells enriched from ER-positive breast cancer cells with different levels of ER-α36 expression.

Task 2: To investigate the function and the underlying mechanisms of ER-α36 in antiestrogen resistance of the ER-negative breast cancer stem/progenitor cells (months 13-24).

2b. To examine the effects of antiestrogens on the enriched stem/progenitor cells from the ER-negative breast cancer cells with different levels of ER-α36 expression and compare to un-enriched ER-negative breast cancer cells to determine if ER-negative stem/progenitor cells are more resistant to antiestrogens (months 13-16, from SOW).

We will finish the experiments to investigate the effects of antiestrogens on the self-renewal and differentiation of the stem/progenitor cells from ER-negative breast cancer cells with knocked-down levels of ER-α36 expression.

2c. To determine if ER-α36 can be used as a novel marker for breast cancer stem/progenitor cells (18-24, from SOW).

We spent a long time to screen for the antibody that is able to specifically recognize ER-α36 but unable to inhibit cell growth. Now with the antibody in place, we will use this antibody to enrich ER-α36-positive cells and to test their stem cell characteristics.

2d. To investigate the underlying mechanisms of ER-α36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens (months 17-24, from SOW).

We will finish our research on the PI3K/AKT signaling pathway in ER-negative breast cancer stem/progenitor cells to examine the possibility of down-regulation of ER-α36 expression or inhibition of the PI3K/AKT pathway to sensitize ER-negative breast cancer stem/progenitor cells to antiestrogens.

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Estrogen Receptor-α Variant, ER-α36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity

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Antiestrogens such as tamoxifen provided a successful treatment for ER-positive breast cancer for the past two decades. However, most breast tumors are eventually resistant to tamoxifen therapy. The molecular mechanisms underlying tamoxifen resistance have not been well established. Recently, we reported that breast cancer patients with tumors expressing high concentrations of ER-α36, a variant of ER-α, benefited less from tamoxifen therapy than those with low concentrations of ER-α36, suggesting that increased ER-α36 concentration is one of the underlying mechanisms of tamoxifen resistance. Here, we investigated the function and underlying mechanism of ER-α36 in tamoxifen resistance. We found that tamoxifen increased ER-α36 concentrations and tamoxifen-resistant MCF7 cells expressed high concentrations of ER-α36. In addition, MCF7 cells with forced expression of recombinant ER-α36 and H3396 cells expressing high concentrations of endogenous ER-α36 were resistant to tamoxifen. ER-α36 downregulation in tamoxifen-resistant cells with the shRNA method restored tamoxifen sensitivity. We also found tamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER-α36 expressing cells. Finally, we found that cells with high concentration of ER-α36 protein were hypersensitive to estrogen; activating ERK phosphorylation at pM range. Our results thus demonstrated that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy and provided a rational to develop novel therapeutic approaches for tamoxifen resistant patients by targeting ER-α36.

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen (TAM) provides a successful option for ER-positive breast cancer patients in the past four decades. However, despite the significant antineoplastic activity of TAM, most breast tumors are eventually resistant to TAM therapy, which largely affects the efficacy of this treatment. Essentially, two forms of TAM resistance occur: de novo and acquired resistance (reviewed in 1–3). Although ER-α absence is the most common de novo resistance mechanism, about 50% ER-positive breast cancer patients with advanced disease do not respond to TAM treatment by the time of diagnosis (reviewed in 2). The exact mechanisms underlying the de novo TAM resistance in these ER-positive tumors are largely unknown. Several mechanisms have been postulated to be involved in the TAM resistance such as increased growth factor signaling, metabolism of TAM by CYP2D6 variants, altered expression of coregulators, mutations of ER-α (reviewed in 3, 4). In addition, most initially responsive breast tumors gradually acquire TAM resistance by loss of TAM responsiveness, the acquired resistance. The mechanisms by which breast tumors lose their TAM responsiveness have not been well established. Breast tumors with acquired TAM resistance frequently retain ER-α expression that would still classify them as ER-positive tumors (3). Therefore, loss of ER-α expression is not a major mechanism driving acquired TAM resistance. Another acquired TAM resistance phenotype has been described in breast cancer xenografts that exhibit a switch from a TAM-inhibitory phenotype to a TAM-stimulated one (5, 6). The agonist activity of TAM in this

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model may be due to the enhanced growth factor signaling that is often associated with acquired TAM resistance (reviewed in 7). However, the molecular mechanism underlying this type of acquired TAM resistance has not been well established.

During development of acquired antiestrogen resistance, breast cancer cells usually undergo adaptive changes in response to inhibitory effects of antiestrogens (8). Adaptive changes also occur in response to aromatase inhibitor therapy in postmenopausal patients or from oophorectomy in premenopausal patients (9, 10). Using a MCF7 breast cancer model system, Santen's group demonstrated that deprivation of estrogen for a prolonged period of time confers these cells hypersensitive to low concentrations of estrogen (8–11). In these hypersensitive cells, 17-β-estradiol (E2) stimulates cell proliferation at pM range while the wild-type cells require nM range E2 to induce cell growth (11). However, the exact molecular events in the development of this "adaptive hypersensitivity" have not been elucidated although up-regulation and membrane localization of ER-α, activation of the non-genomic estrogen signaling, as well as induction of c-Myc and c-Myb have been proposed to be involved in this process (8, 12).

Previously, our laboratory identified and cloned a variant of ER-α, ER-α36, which has a molecular weight of 36-kDa (13, 14). The transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α gene (15). This ER-α differs from the original 66 kDa ER-α (ER-α66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains, and partial ligand-binding domain (13). ER-α36 is mainly localized near the plasma membrane and mediates membrane-initiated estrogen signaling (14). We also found that the breast cancer patients with tumors expressing high concentrations of ER-α36 benefited less from TAM therapy than those with low concentrations of ER-α36 (16), suggesting that increased ER-α36 concentration is one of the underlying mechanisms of TAM resistance. Recently, we also reported that ER-α36 is able to mediate agonist activity of TAM and ICI 182, 780 (17, 18) such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways, indicating these antiestrogens may loss their growth inhibitory activities in cells with increased ER-α36 expression.

Based on these observations, we hypothesized that ER-α36 is involved in TAM resistance. Using ER-positive breast cancer MCF7 cells with different concentrations of ER-α36 as model systems, we investigated ER-α36 function in TAM resistance. Here, we present evidence to demonstrate that ER-α36 plays an important role in TAM resistance presumably through mediating agonist activity of TAM and estrogen hypersensitivity.

Materials and Methods

Chemicals and Antibodies

17β-estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho-p44/42 ERK (Thr202/Tyr204) (197G2) mouse monoclonal antibody (mAb), anti-p44/42 ERK (137F5) rabbit mAb, anti-phospho-AKT (Ser473) (D9E) rabbit mAb and anti-AKT (pan) (C67E7) Rabbit mAb, anti-EGFR and HER2 antibodies were purchased from Cell Signaling Technology (Boston, MA). Antibodies of ER-α66 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ER-α36 antibody was generated by the custom service provided by the Pacific Immunology Corp. (Ramona, CA) using the last 20 amino acids of the ER-α36 encoded by the exon 9 that is unique to ER-α36 as an immunogen. The produced antibody was purified with an affinity column made of immunogen peptides. The antibody was characterized and validated with a number of experiments including immpunoprecipitation, immunofluorescence staining, Western blot analysis. The antibody specifically recognizes ER-α36 and does not cross react with ER-α66.

Cell culture and establishment of stable cell lines

The MCF7 cell line (ATCC) and its derivatives as well as H3396 cells (a kind gift from Dr. Leia Smith of Seattle Genetics) were maintained at 37°C in a 10% CO_2 atmosphere in IMEM without phenol red and 10% fetal calf serum. To establish stable cell lines with knocked-down concentrations of ER-α36, we constructed an ER-α36 specific shRNA expression vector by cloning the DNA oligonucleotides 5′-GATGCCAATAGGTACT-GAATTGATATCCGTTCAGTACCTATT GGCAT-3′ from the 3′UTR of ER-α36 gene into the pRNAT-U6.1/Neo expression vector from GenScript Corp. Briefly, cells transfected with the empty expression vector and ER-α36 shRNA expression vector were selected with 500 μg/ml G418 for three weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER-α36 expression with Western blot analysis and retained for experiments. For ERK1/2 and AKT activation assays, cells were treated with vehicle (ethanol) and indicated concentrations of tamoxifen or E2.

To examine cell growth in the presence or absence of antiestrogens, cells maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) were treated with different concentrations of tamoxifen, 17β-estradiol or ethanol vehicle as a control. The cells were seeded at 1×10^4 cells per dish in 60 mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea) after seven days. Five dishes were used for each treatment and experiments were repeated at least three times.

Western blot analysis

For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM

NaF) plus the protease and phosphatase inhibitors (Sigma). The protein amounts were measured using the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA). The same amounts of the cell lysates were boiled for 5 min in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). All Western blot experiments were performed at least three times. Band densities on developed films were measured and analyzed using Quantity One 1-D Analysis Software Version 4.6.7 (BIO-RAD Laboratories).

Statistical analysis

Data were summarized as the mean ± standard error (SE) using the GraphPad InStat software program. Tukey-Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P < .05$.

Results

Tamoxifen treatment induces ER-α36 protein concentration in ER-positive breast cancer MCF7 cells

Previously, our laboratory identified and cloned a 36 kDa variant of ER-α, ER-α36 that functions differently from the 66 kDa full-length ER-α, ER-α66 (13, 14). Using an ER-α36 specific antibody, we further found that ER-α36 is highly expressed in established ER-negative breast cancer cells while weakly expressed in ER-positive breast cancer cells such as MCF7 (14). In order to investigate ER-α36 function in the activities of antiestrogens, we first examined whether TAM influences ER-α36 expression in MCF7 cells. The steady state concentration of ER-α36 protein in MCF7 cells treated with 1 μM of TAM for different time periods or different concentrations of TAM was examined with Western blot analysis. After TAM treatment, ER-α36 protein concentration was increased in MCF7 cells in a time and concentration dependent manner (Figure 1), indicating that TAM is able to increase ER-α36 concentration in ER-positive breast cancer MCF7 cells.

TAM-resistant ER-positive breast cancer MCF7 cells express high concentration of ER-a36 protein

To examine the possible involvement of ER-α36 in development of acquired TAM resistance, we cultured MCF7 cells in the presence of TAM (1 μM) for six months and pooled all surviving cells to establish a cell line MCF7/TAM. This cell line exhibited resistance to the growth inhibitory activity of TAM compared to the parental cells and TAM at 1 μM even acted as an agonist in MCF7/TAM cells (Figure 2A). Western blot analysis revealed that MCF7/TAM cells expressed higher concentration of ER-α36 protein compared to the MCF7 parental cells, while ER-α66 protein concentration was without significant change (Figure 2B), suggesting that MCF7 cells gained ER-α36 expression during development of acquired TAM

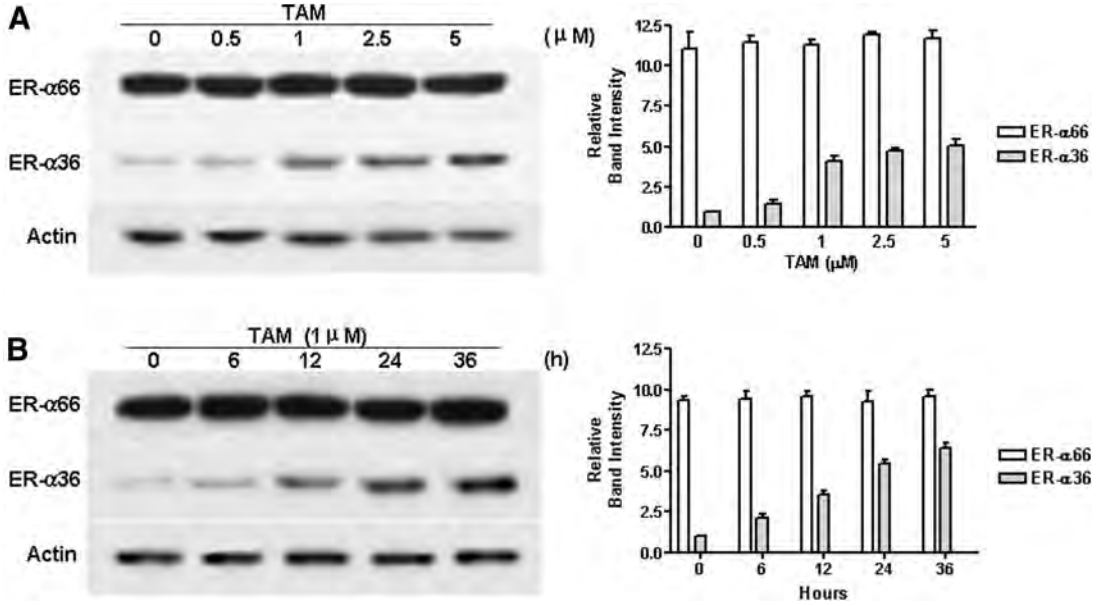

Figure 1. Tamoxifen treatment increases concentrations of ER-α36 protein. A. Western blot analysis of the concentrations of ER-α36 and 66 proteins in ER-positive breast cancer MCF7 cells treated with indicated concentrations of tamoxifen (TAM) for 12 h. B. Western blot analysis of MCF7 cells treated with 1 μM of TAM for indicated hours (h). All experiments were done at least three times and representative results are shown. Relative band intensities were obtained by quantitative densitometric scanning of autoradiographc signals and are shown with the ER-α36 band densities from the cells treated with vehicle (ethanol, 0 μM of TAM) or at 0 h that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

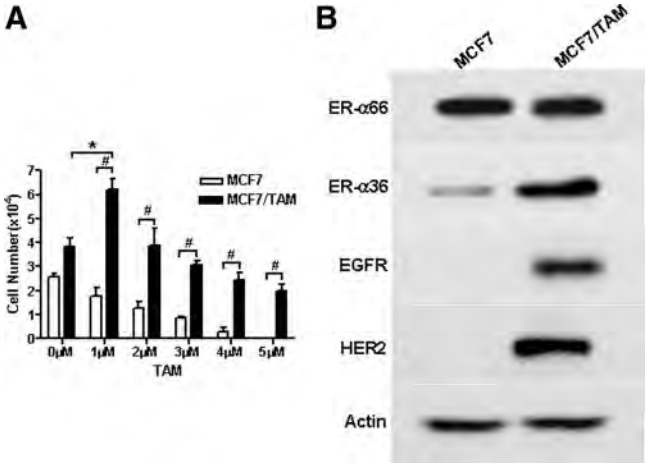

Figure 2. Tamoxifen resistant ER-positive breast cancer MCF7 cells express high concentrations of endogenous ER-α36. A. ER-positive breast cancer MCF7 cells and tamoxifen resistant MCF7 cells (MCF7/TAM) cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and surviving cells were counted. The columns represent the means of three experiments; bars, SE. *, $P < .05$ for MCF/TAM cells treated with vehicle vs cells treated with 1 μM of TAM. #, $P < .01$ for MCF/TAM cells vs MCF cells treated with indicated concentrations of TAM. B. Western blot analysis of the expression concentrations of ER-α36 and 66, EGFR and HER2 in MCF7 and MCF7/TAM cells.

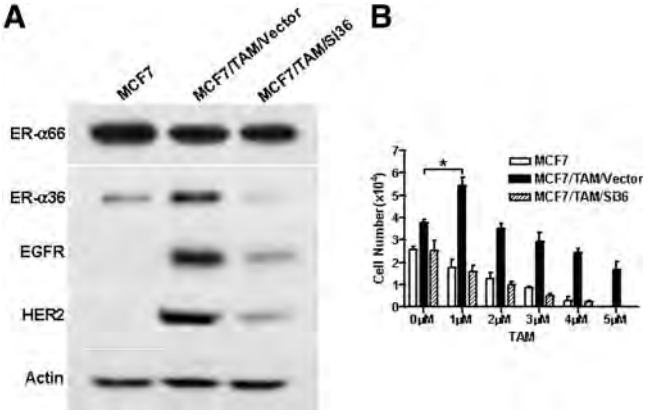

Figure 3. ER-α36 is involved in tamoxifen resistance. A. Western blot analysis of the concentrations of ER-α36 and 66, EGFR and HER2 proteins in MCF7 cells, MCF7/TAM cells transfected with the empty expression vector (MCF/TAM/Vector) and MCF7/TAM cells transfected with an ER-α36 specific shRNA expression vector (MCF7/TAM/Si36). B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and the numbers of surviving cells were determined. The columns represent the means of three experiments; bars, SE. *, $P < .05$ for MCF/TAM/Vector cells treated with vehicle vs cells treated with 1 μM of TAM.

resistance. MCF/TAM cells also expressed increased concentrations of EGFR/ErbB1 and HER2/ErbB2 proteins (Figure 2B), indicating MCF7/TAM cells also gained expression and signaling of the EGFR/HER2 pathway.

High concentration of ER-α36 protein confer TAM resistance

To confirm that elevated concentration of ER-α36 protein is involved in TAM resistance, we sought to down-regulate ER-α36 expression in MCF7/TAM cells using the shRNA approach. We established a cell line with knocked-down concentration of ER-α36 protein (MCF7/TAM/Si36) from MCF7/TAM cells using the shRNA method as evidenced by Western blot analysis (Figure 3A). We also noticed that the concentrations of EGFR and HER2 were also decreased in MCF7/TAM/Si36 cells (Figure 3A). ER-α36 knockdown restored the sensitivity of MCF7/TAM cells to the growth inhibitory effects of TAM to a level similar to parental MCF7 cells (Figure 3B). Our data thus suggested that elevated ER-α36 concentration is involved in development of acquired TAM resistance.

To further confirm elevated ER-α36 expression contributes to TAM resistance, we introduced recombinant ER-α36 into MCF7 cells that express high concentration of ER-α66 protein but lower concentration of ER-α36 to establish a stable cell line, MCF7/ER36. Western blot analysis confirmed that recombinant ER-α36 protein was highly expressed in MCF7/ER36 cells compared to the control MCF7 cells transfected with the empty expression vector (Figure 4A). We also observed that the EGFR expression was strongly increased while the HER2 expression was weakly increased in MCF7/ER36 cells (Figure 4A), consistent with our previous report that ER-α36 stabilizes EGFR protein (19). When MCF7/ER36 cells were treated with different concentrations of tamoxifen, these cells are more resistant to the growth inhibitory effects of TAM compared to the control MCF7 cells (Figure 4B), indicating that increased ER-α36 concentration is one of the underlying mechanism of tamoxifen resistance. We also found a breast cancer cell line H3396 that expressed high concentration of endogenous ER-α36 protein (Figure

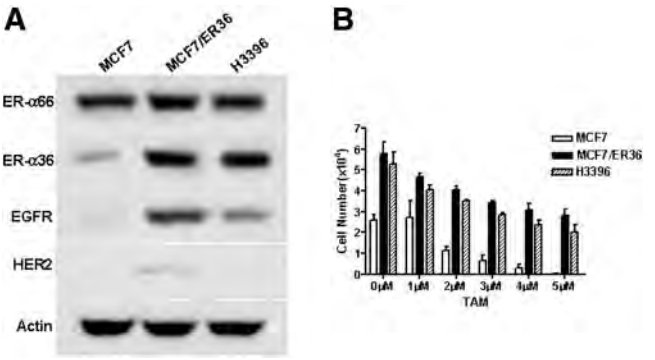

Figure 4. ER-positive breast cancer cells with elevated concentrations of ER-α36 protein are resistant to tamoxifen. A. Western blot analysis of the lysates from ER-positive breast cancer MCF7 cells, MCF7 cells with forced expression of ER-α36 (MCF7/ER36) and H3396 cells with high concentrations of endogenous ER-α36 protein. The concentrations of EGFR and HER2 proteins are also shown. B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and the numbers of surviving cells were counted. The columns represent the means of three experiments; bars, SE.

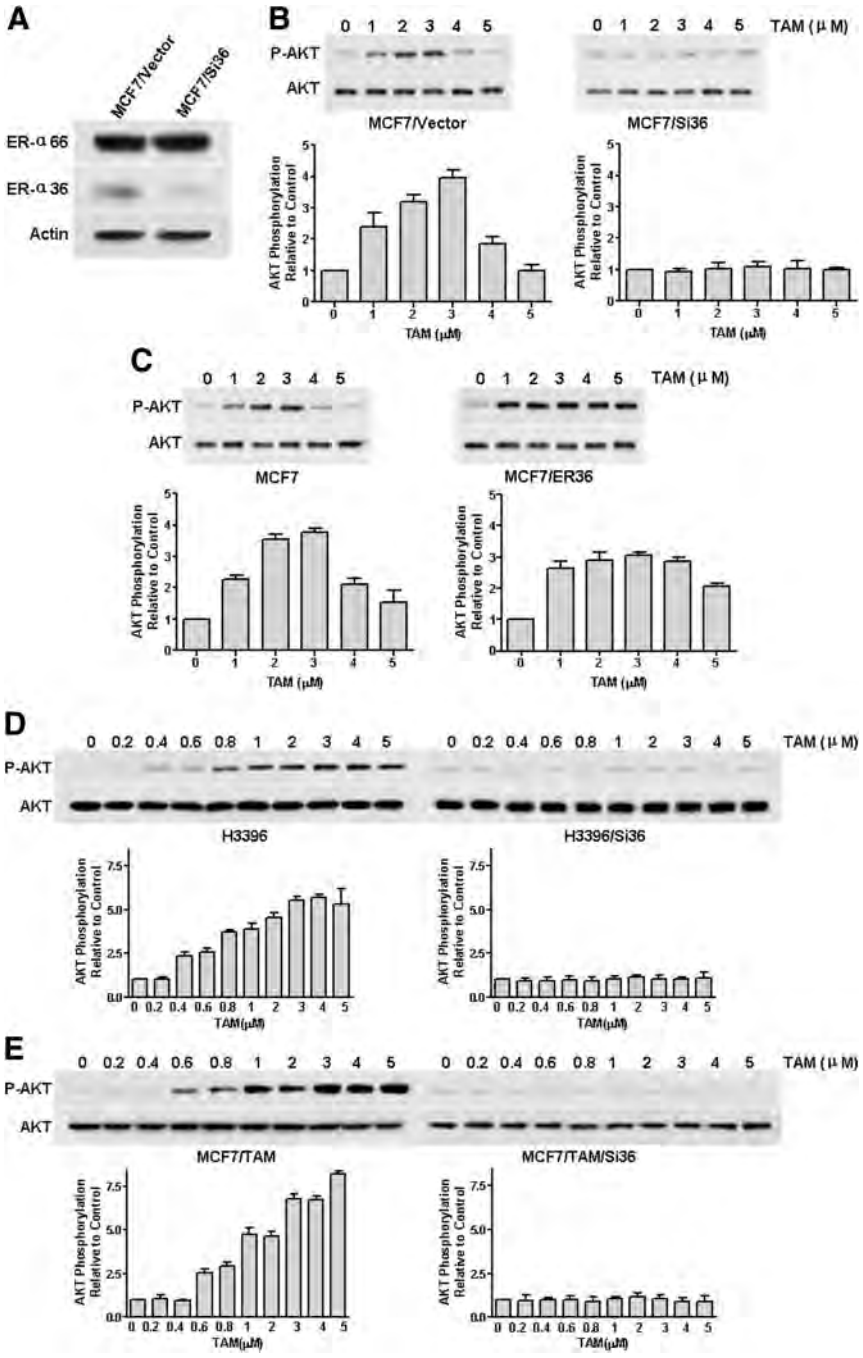

Figure 5. ER-α36 mediates tamoxifen-induced phosphorylation of the AKT kinase in ER-positive breast cancer MCF7 cells. A. Western blot analysis of ER-α36 and 66 protein concentrations in MCF7 cells transfected with an empty expression vector (MCF7/Vector) and MCF7 cells transfected with the ER-α36 specific shRNA expression vector (MCF7/Si36). B. Western blot analysis of phosphorylation of AKT in MCF7/Vector and MCF7/Si36 cells treated with indicated concentrations of tamoxifen (TAM) using phospho-specific or nonspecific AKT antibodies. C. Western blot analysis of phosphorylation of AKT in MCF7 and MCF7/ER36 cells treated with different concentrations of TAM. D. Western blot analysis of phosphorylation of AKT in H3396 and H3396/Si36 cells treated with indicated concentrations of TAM using phospho-specific or nonspecific AKT antibodies. D. Western blot analysis of phosphorylation of AKT in MCF7/TAM and MCF7/TAM/Si36 cells treated with indicated concentrations of TAM. All experiments were done at least three times and representative results are shown. AKT phosphorylation intensities were obtained by quantitative densitometric scanning of autoradiographc signals obtained with the phospho-specific AKT antibody and normalized with the signals obtained by the phospho-nonspecific AKT antibody. The relative band intensities are shown with the band densities from the cells treated with vehicle (ethanol, 0 μM of TAM) that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

4A). Like MCF7/ER36 cells, H3396 cells were more resistant to the growth inhibitory effects of TAM compared to the control MCF7 cells (Figure 4B).

Tamoxifen induces AKT activation in cells expressing ER-a36

Previously, we found that TAM elicited agonist activities such as activation of the MAPK/ERK and the PI3K/AKT pathways in ER-α36 expressing endometrial cells (15, 16). We sought to determine whether ER-α36 mediates agonist activity of TAM in cells with high concentrations of ER-α36. We first treated MCF7 cells with different concentrations of TAM and the AKT phosphorylation was measured with Western blot analysis. In control MCF7 cells transfected with the empty expression vector (MCF7/Vector), we found that at lower concentrations from 1 to 3 μM, TAM induced the AKT phosphorylation while at 4–5 μM failed to do so (Figure 5B). However, TAM at different concentrations failed to induce AKT activation in MCF7 cells with ER-α36 knocked-down (Figure 5 A & B), indicating ER-α36 mediates agonist activity of TAM. However, in MCF7/TAM, H3396 and MCF7/ER36 cells, TAM potently induced the AKT phosphorylation even at 4–5 μM (Figure 5C, D, E). In MCF7/TAM and H3396 cells, TAM induced AKT phosphorylation at 0.4 μM (Figure 5D, E). To further confirm the role of ER-α36 in the agonist activity of TAM, we also used MCF/TAM and H3396 cells with knocked-down concentrations of ER-α36 protein, and found that TAM failed to induce AKT phosphorylation in these cells (Figure 5D, E). Taken together, these results demonstrated that tamoxifen acts as an agonist to induce AKT phosphorylation in cells expressing ER-α36,

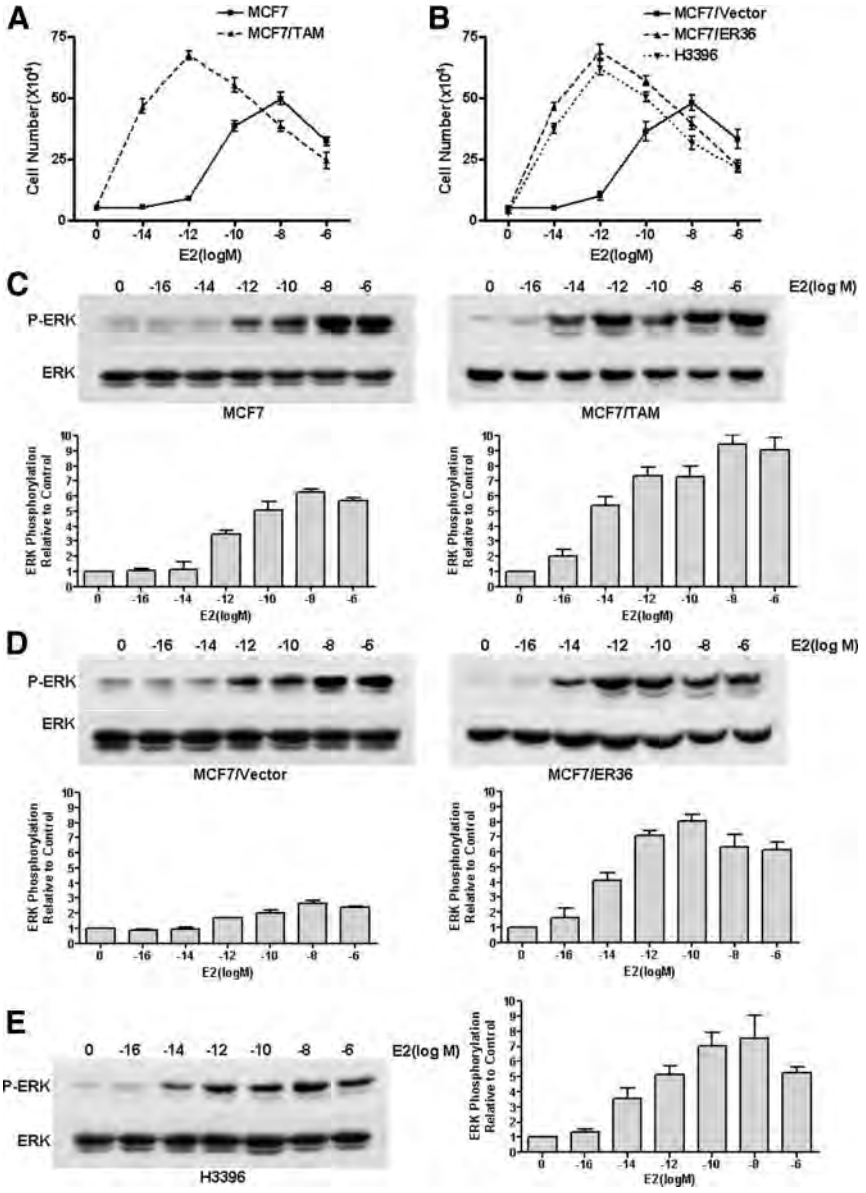

Figure 6. ER-α36 is involved in estrogen hypersensitivity. A. ER-positive breast cancer MCF7 cells and MCF7/TAM cells were treated with indicated concentrations of 17β-estradiol (E2) for seven days and cell number was counted. Each point represents the means of three experiments; bars, SE. B. MCF7 cells transfected with the empty expression vector (MCF7/Vector) and ER-α36 expression vector (MCF7/ER36) as well as H3396 cells were treated with indicated concentrations of E2 for seven days and cell number was determined. Each point represents the means of three experiments; bars, SE. C, D, E. Estrogen induces ERK activation in different cell lines. Western blot analysis of the lysates from different cells treated with indicated concentrations of E2 for 30 min using the phospho-specific or nonspecific ERK1/2 antibodies. All experiments were done at least three times and representative results are shown. ERK phosphorylation levels were obtained by quantitative densitometric scanning of autoradiographc signals obtained with the phospho-specific ERK antibody and normalized with the signals obtained by the phospho-nonspecific ERK antibody. The relative band intensities are shown with the band densities from the cells treated with vehicle (ethanol, 0 M of E2) that were arbitrarily set as 1. The columns represent the means of three experiments; bars, SE.

ER-a36 expressing breast cancer cells exhibit estrogen hypersensitivity

Previously, it was reported that cells deprived of estrogen for a long-term exhibited hypersensitivity to estrogens (8). We decided to examine whether ER-α36 is involved in development of estrogen hypersensitivity. MCF7/TAM cells were treated with different concentrations of 17β-estradiol (E2) for seven days. We found that E2 stimulated stronger proliferation in these cells compared to the parental MCF7 cells (Figure 6A). In addition, MCF7/TAM cells exhibited hypersensitivity to E2; at pM range, E2 stimulated proliferation of MCF/TAM cells while E2 stimulated proliferation of the parental MCF7 cells at nM range (Figure 6A). We also found that MCF7/ER36 cells that express recombinant ER-α36 and H3396 cells with high concentrations of endogenous ER-α36 protein also exhibited estrogen hypersensitivity (Figure 6B), suggesting that ER-α36 is involved in estrogen hypersensitivity.

We then examined E2-induced phosphorylation of the MAPK/ERK1/2, a typical nongenomic estrogen-signaling event, in different cell lines. Cells were treated with E2 at different concentrations for 30 min, and Western blot analysis with a phospho-specific ERK1/2 antibody was performed. Figure 6C shows that E2 elicited ERK phosphorylation in MCF/TAM cells in a dos-dependent manner starting at a very low concentration, 1×10^{-14} M/L, while in the parental MCF7 cells and MCF/Vector cells, ERK activation requires E2 at 1×10^{-12} M/L (Figure 6C, D). A similar hypersensitivity was also observed in MCF7/ER36 and H3396 cells (Figure 6D & E); E2 induced ERK phosphorylation at 1×10^{-14} M/L. Our data thus suggested that increased concentration of ER-α36 protein is one of the mechanisms underlying estrogen hypersensitivity.

which provides an explanation to the involvement of ER-α36 in TAM resistance.

Discussion

Tamoxifen therapy is the most effective treatment for advanced ER-positive breast cancer, but its effectiveness is limited by high rate of de novo resistance and resistance acquired during treatment. Many studies were conducted to understand the molecular pathways responsible for the de novo and acquired tamoxifen resistance, and have revealed that multiple signaling molecules and pathways are involved in tamoxifen resistance. All of these pathways often bypass the requirement of estrogen signaling pathway for growth of ER-positive breast cancer cells. Previously, we reported that the breast cancer patients with tumors expressing high concentrations of endogenous ER-α36 less benefited from tamoxifen therapy than those with low concentrations of ER-α36 (16), suggesting elevated concentration of ER-α36 protein may be a novel mechanism underlying both de novo and acquired tamoxifen resistance.

Here, we showed that tamoxifen treatment induced ER-α36 expression and tamoxifen resistant MCF7/TAM cells selected with long-term cultivation in the presence of tamoxifen expressed elevated concentration of ER-α36 protein. We also showed that MCF7 cells with forced ER-α36 expression and H3396 cells that express high concentration of endogenous ER-α36 protein were relatively more resistant to tamoxifen compared to MCF7 cells. Downregulation of ER-α36 expression, however, was able to restore tamoxifen sensitivity in MCF/TAM and H3396 cells, indicating that increased ER-α36 concentration is one of the molecular mechanisms by which ER-positive breast cancer develops tamoxifen resistance.

Previously, we found that antiestrogens TAM and ICI 182, 780 failed to block ER-α36-mediated nongenomic estrogen signaling (14). Here we showed that TAM exhibited a biphasic activation of the AKT kinase in tamoxifen-sensitive MCF7 cells; increasing AKT phosphorylation at low concentrations and failed to do so at higher concentrations. However, in cells with high concentrations of ER-α36 protein, TAM still activates the AKT kinase at higher concentrations, consistent with our recent report that ER-α36 mediates agonist activities of both TAM and ICI 182, 780 (18). Recently, loss of p21 (CDKN1A), a cyclin-dependent kinase inhibitor was found to be associated with the agonist activity of tamoxifen (20). Likewise, inhibition of p27 (CDKN1B), another cyclin-dependent kinase inhibitor, by Src has been associated with a tamoxifen-resistance phenotype (21). Both p21 and p27 are phosphorylated by the AKT kinase and this phosphorylation banishes both p21 and p27 from the cell nucleus and keeps them in the cytoplasm (22, 23). Thus, loss of expression and function, and relocalization of either of two G1-checkpoint CDK inhibitors after AKT phosphorylation can lead to TAM resistance. We found that in TAM sensitive MCF7 cells, TAM downregulated p27 phosphorylation and increased concentration of p27 protein whereas TAM upregulated p27 phosphorylation and decreased concentration of p27 protein (Zhang et al. unpublished data). Our results suggested that ER-α36 mediated agonist activity of TAM such as activation of the PI3K/AKT signaling is important for ER-α36 function in TAM resistance.

Previously, another acquired TAM resistance phenotype has been described in a human breast cancer xenograft model that exhibits a switch from a TAM-inhibitory phenotype to a TAM-stimulated one. Some breast cancers may be initially inhibited by TAM, and later become dependent on TAM for proliferation (24–26). These xenogafts also retain the ability to be stimulated by estrogens (24–26). In the current study, we found that 1 μM of TAM stimulated proliferation of MCF7/TAM cells while downregulation of ER-α36 expression in these cells diminished TAM-stimulation. In addition, these TAM resistant cells retained estrogen responsiveness, and even showed estrogen hypersensitivity. Our results thus suggested that elevated ER-α36 concentration is involved in this type of TAM resistance. It also worth noting that the TAM at 1 μM failed to stimulate proliferation of MCF7/36 cells that express recombinant ER-α36 and H3396 that express endogenous ER-α36 (Figure 4B). The exact mechanism for this is not known. We observed that TAM-resistant MCF7/TAM cells also gained expression of the growth factor receptors EGFR and HER2 while MCF7/ER36 cells mainly increased the concentration of EGFR protein and H3396 cells only express modest concentration of EGFR. Thus, it is possible that increased expression or signaling of the HER2 receptor in MCF7/TAM cells contributes to the TAM-stimulated proliferation in MCF7/TAM cells. Intriguingly, the expression of both EGFR and HER2 were downregulated in MCF7/TAM/Si36 cells, consistent with our recent reports there are positive regulatory loops between ER-α36 and the EGFR/HER2 expression; ER-α36 stabilizes EGFR protein and activates HER2 promoter activity while the signaling of EGFR/HER2 induces ER-α36 expression (19, 31).

Previously, it has been reported that physiological concentrations of E2 exhibit antitumor activity in a TAM-stimulatory MCF7 cell model that was generated by serial transplantation of TAM resistant tumors in the continuous presence of TAM (27). Based on the laboratory studies, it was recently proposed that physiological concentration of estrogen could be used as a therapeutic approach for these TAM resistant patients (28, 29). However, the molecular mechanisms underlying this paradox-

ical phenomenon have not been well elucidated. It is known that estrogen stimulates growth of ER-positive breast cancer cells in a biphasic growth curve; stimulating cell proliferation at low concentrations while failing to stimulate or even inhibiting cell growth at higher concentrations. Our results presented here that elevated ER-α36 concentration rendered cells hypersensitive to E2; shifting the biphasic growth curve to the left. Thus, in cells expressing high concentrations of ER-α36 protein, physiological concentrations of E2 may fail to stimulate proliferation or even inhibit proliferation. Our data thus provided a molecular explanation to the paradoxical phenomenon that some TAM resistant tumors are simulated by TAM but inhibited by estrogen.

Previously, it was reported that long-term estrogen deprivation with hormonal therapy resulted in "adaptive" changes of breast cancer cells; making these cells hypersensitive to estrogen (8, 11). Recently, we reported that ER-α36 concentration is significantly increased in normal osteoblasts cells from menopausal women (30), suggesting that ER-α36 expression is elevated in response to low concentration of estrogen in menopausal women. Our current data showed that E2 induced ERK phosphorylation and stimulated proliferation at pM range in cells with high concentration of ER-α36 protein while at nM range in cells with low concentration of ER-α36. Thus, our results indicated that gained ER-α36 expression is one of the "adaptive" changes in breast cancer cells after a long-term estrogen deprivation resulted from antiestrogen treatment.

In summary, here we provided evidence to demonstrate that ER-α36 is a novel and important player in normal and abnormal estrogen signaling, and ER-α36 is involved in many physiological and pathological processes regulated by estrogen signaling. Our findings that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape the antiestrogen therapy provided a rational to develop novel therapeutic approaches for antiestrogen resistant patients by targeting ER-α36.

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Broussoflavonol B Restricts Growth of ER-negative Breast Cancer Stem-like Cells

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Abstract. *Accumulating experimental and clinical evidence has indicated that tumor-initiating or cancer stem-like cells are a sub-population of tumor cells capable of initiating and driving tumor growth, and cancer stem-like cells are resistant to most current cancer therapies, including chemo- and radiation therapy. More effective targeted-therapeutic approaches are urgently needed to eliminate cancer stem-like cells. Here, we report that broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (broussonetia papyrifera), exhibited potent growth inhibitory activity towards estrogen receptor (ER)-negative breast cancer SK-BR-3 cells at sub-micromolar concentrations. Broussoflavonol B more potently inhibited growth and induced differentiation of stem-like SK-BR-3 cells-compared to the anti-estrogen tamoxifen. In addition, broussoflavonol B treatment also reduced the steady, state levels of the Human epidermal growth factor receptor-2 (HER2) and ER-α36, a variant of ER-α. Our results, thus, indicate that broussoflavonol B is a potent growth inhibitor of ER-negative breast cancer stem-like cells and provide a rationale for pre-clinical and clinical evaluation of broussoflavonol B for breast cancer therapy.*

Tumor-initiating, or cancer stem-like, cells are a sub-population of tumor cells capable of initiating and driving tumor growth. Accumulating experimental and clinical evidence supports the hypothesis that breast cancer arises from a sub-population of mammary stem/progenitor cells that possess the ability to self-renew (1-5). Al-Hajj *et al*. enriched a $CD44^+/CD24^{-/low}$ cell population from human breast cancer that displayed cancer stem/progenitor cell properties and was capable of forming tumors in immunocompromised mice with higher efficiency than in cells with alternative phenotypes (6). Later, aldehyde dehydrogenase (ALDH)-1 expression and its activity were identified to be a marker for breast cancer stem/progenitor cells; fewer ALDH1-positive tumor cells than $CD44^+/CD24^{-/low}$ tumor cells are required to generate tumors *in vivo* (7). Breast cancer with $ALDH1^{high}$ cancer stem-like cells are associated with more aggressive tumor phenotypes such as these with estrogen receptor negativity, high histological grade, Human epidermal growth factor receptor-2 (HER2) positivity, as well as poor prognosis (8).

Accumulating evidence has indicated that cancer stem-like cells are resistant to many current cancer therapies, including chemo- and radiation therapy as well as hormone therapy (9-13). This suggests that many cancer therapies, while killing the bulk of tumor cells, may eventually fail since they do not eliminate cancer stem-like cells that survive to regenerate new tumors. Thus, novel and effective therapeutic agents that target cancer stem-like cells are urgently needed.

Previously, we identified and cloned a 36-kDa variant of ER-α, ER-α36, that is mainly expressed at the plasma membrane and in the cytoplasm, and mediates non-genomic estrogen signaling (14, 15). ER-α36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER-α (ER-α66), consistent with the fact that ER-α36 has no intrinsic transcriptional activity (15). ER-α36 is generated from a promoter located in the first intron of the *ER-α66* gene (16), indicating that ER-α36 expression is regulated differently from ER-α66, consistent with the findings that ER-α36 is expressed in specimens from ER-negative breast cancer and established ER-negative breast cancer cells that lack ER-α66 expression (17, 18). ER-α36 is highly expressed in ER-negative SK-BR-3 breast cancer cells and positively regulates HER2 expression in these cells (19). ER-α36 expression is required for maintenance of the ALDH1-positive stem-like SK-BR3 cells; knockdown of ER-α36 expression with the short hairpin RNA (shRNA) method dramatically reduced the population of ALDH1-positive cells (19). Thus, ER-α36-mediated signaling plays an important role in maintenance of ER-negative breast cancer stem-like cells, and down-regulation of ER-α36 expression may provide a novel approach to inhibit proliferation of ER-negative breast cancer stem-like cells.

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Key Words: Broussoflavonol B, estrogen receptor, cancer stem-like cells.

Recently, we reported that several flavonoid derivatives purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) (L.) were able to down-regulate ER-α36 expression (20). In the present study, we examined the growth inhibitory activity of the most potent ER-α36 down-regulator broussoflavonol B (5,7,3',4'-tetrahydroxy-3-methoxy-6,8-diprenylflavone) from the bark of the Paper Mulberry tree on SK-BR-3 cells.

Materials and Methods

Chemicals and reagents. Broussoflavonol B (99.8% pure) was obtained from Beijing Shenogen Pharma Group (Beijing, China). Antibody to HER2 was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody to ER-α36 was generated and characterized as described elsewhere (18). Antibodies to β-actin, CD10 (H-321), and cytokeratin-18 (DC-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. SK-BR-3 cells were purchased from the American type culture collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 24 h. All cells were maintained at 37˚C and in 5% CO_2 in a humidified incubator. The ALDEFLUOR kit (StemCell Technologies, Vancouver, BC, Canada) was used to examine the cell population with high ALDH1 enzymatic activity according to the manufacturer's instructions using a FACSCalibur flow cytometer (BD-Biosciences, San Jose, CA, USA).

Cell growth assay. Cells in phenol red-free medium were seeded into 35 mm dishes at 5×10^4 cells/dish. After 24 h, broussoflavonol B (0. 1 to 1 μM) or tamoxifen (0.1 to 1 μM) were added and cells incubated for another seven days. The vehicle dimethyl sulfoxide (DMSO) was used as a control. Cells were then trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Soul, Korea). Three dishes were used for each concentration tested and the experiments were repeated three times.

For the growth assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 6-well plates (Corning Incorporated, Corning, NY, USA) at 10,000 cells/ml and cultured in the stem cell culture medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1X B27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). Different concentrations of broussoflavonol B or tamoxifen were added and cells were then incubated for seven days. Cells were then collected, washed with phosphate buffered saline (PBS) and trypsinized to dissociate cells, then counted using the ADAM automatic cell counter (Digital Bio) or examined for ALDH1 positive cells using the ALDEFLUOR kit.

Indirect immunofluorescent staining. Treated cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and then permeabilized in 0.5% Triton X-100 for 15 min. After washing with PBS, cells were incubated for 1 h at room temperature with different primary antibodies followed by extensive washing with PBS. The cells were then incubated for over 1 h at room temperature with secondary antibody Alexa 488-conjugated rabbit anti-mouse (Molecular Probes, Carlsbad, CA, USA) diluted 1:100 in PBS. Cells were washed with PBS and mounted with 10 mg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) in aqueous mountant (Dako, Carpinteria, CA, USA) and photographed using a Zeiss fluorescence microscope (Carl Zeiss).

Western blot assay. Cells were washed with cold PBS twice and lysed with the radioimmunoprecipitation assay (RIPA) buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma-Aldrich). The cell lysates were boiled for five minutes in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies, which were then visualized with the corresponding secondary antibodies and ECL kit (Thermo Scientific, Rockford, IL, USA).

Cell differentiation assay. For the differentiation assay of cancer stem-like cells, SK-BR-3 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell culture medium for seven days. Broussoflavonol B or tamoxifen were then added and cells were incubated for another three days. Cells were collected, washed with PBS and cytospinned onto slides. Cytospinned slides were stained with indirect immunofluorescent staining using antibodies to CD10 and CK18. Five hundred cells were assessed for CD10 or CK18 positivity under fluorescence microscopy (Nikon, Eclipss E600, Melville, NY, USA), and the percentage of cells positive for these markers was calculated.

Statistical analysis. Data are summarized as the means±standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student-Newman-Keuls testing and the significance was accepted for *p*-values less than 0.05.

Results

Broussoflavonol B inhibits proliferation of ER-negative SK-BR-3 breast cancer cells. Recently, we reported that broussoflavonol B (Figure 1A) was able to down-regulate ER-α36 expression in ER-positive MCF7 breast cancer cells (20). Since ER-α36 plays an important role in malignant growth in ER-negative breast cancer SK-BR-3 cells (19), we decided to test whether broussoflavonol B influences their growth. SK-BR-3 cells were incubated with different concentrations of broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the numbers of surviving cells were counted. We found that broussoflavonol B potently inhibited growth of SK-BR-3 cells, while tamoxifen had no effect (Figure 1B), consistent with the fact that anti-estrogens have less or no effect on the growth of ER-negative breast cancer cells.

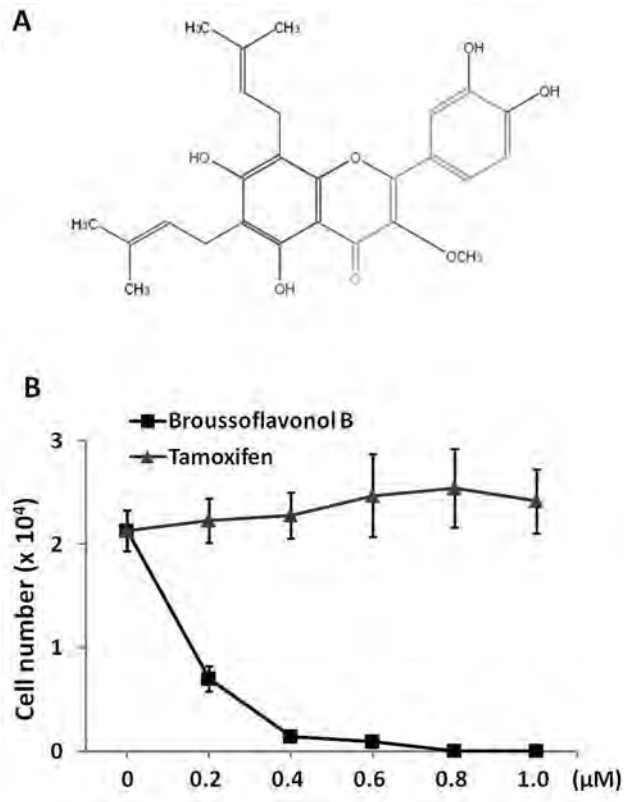

Figure 1. *Broussoflavonol B inhibits growth of estrogen receptor (ER)-negative SK-BR-3 breast cancer cells. A. The chemical structure of broussoflavonol B (5,7,3',4'-tetrahydroxy-3-methoxy-6,8-diprenylflavone). B. Broussoflavonol B inhibits growth of SK-BR-3 breast cancer cells. Cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with vehicle dimethyl sulfoxide (DMSO, 0), 0.2, 0.4, 0.6, 0.8 and 1.0 μM of broussoflavonol B or tamoxifen for seven days before cells were trypsinized and counted. Three dishes were used for each concentration in all of the experiments and all experiments were repeated three times. Each point represents the mean±S.D. of three independent experiments.*

Broussoflavonol B down-regulates expression of both ER-a36 and HER2 in SK-BR-3 cells. To probe the underlying mechanisms by which broussoflavonol B inhibits the growth of SK-BR-3 cells, we decided to determine whether broussoflavonol B influences ER-α36 expression in SK-BR-3 cells. Western blot analysis indicated that broussoflavonol B treatment potently down-regulated ER-α36 expression in a dose- and time-dependent manner (Figure 2A and B), whereas the anti-estrogen tamoxifen modestly increased the levels of ER-α36 expression in SK-BR-3 cells (Figure 2C). Previously, we found that a positive regulatory loop between ER-α36 and HER2 is critical for growth of SK-BR-3 cells; they positively regulate each other's promoter activity (19). We also found that HER2 expression was dramatically down-regulated in SK-BR-3 cells treated with broussoflavonol B (Figure 2D). Thus, our data indicate that disruption of the positive regulatory loop of ER-α36 and HER2 is one of the mechanisms by which broussoflavonol B inhibits growth of these cells.

Broussoflavonol B inhibits the growth of breast cancer stem/progenitor cells. Accumulating evidence has demonstrated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (1-6). Breast cancer stem/progenitor cells are involved in resistance to chemo-and radiation therapies (9-13). Previously, we reported that ER-α36 expression is required for maintenance of the ALDH1-positive stem-like cells in SK-BR-3 cells; knockdown of ER-α36 expression with the short hairpin (sh) RNA method significantly reduced the population of ALDH1-positive SK-BR-3 cells (19). We decided to test the effects of broussoflavonol B on stem-like SK-BR-3 cells. With this aim, we cultured SK-BR-3 cells in a low-serum stem/progenitor cell culture medium and in ultralow-attachment dishes, which enriches the breast cancer stem-like cells. These stem-like cells were then treated with different concentrations of broussoflavonol B or tamoxifen for seven days and cell numbers were counted. We found that broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had a lesser effect (Figure 3A). We then tested the effects of broussoflavonol B on the ALDH1-positive SK-BR-3 cell population. ALDH expression or its activity has been used as a marker for breast cancer stem/progenitor cells (7). We first treated SK-BR-3 cells with 1 μM and 5 μM of broussoflavonol B or tamoxifen for seven days, and the ALDH1-positive cells from the remaining SK-BR-3 cells were analyzed, using the ALDEFLOUR kit and flow-cytometry. We found that treatment of SK-BR-3 cells with broussoflavonol B significantly reduced the population of ALDH1-positive cells while tamoxifen at 1 μM weakly but significantly increased the ALDH1-positive cell population (Figure 3B). These results indicate that the ALDH-high cells, *i.e.* breast cancer stem-like cells, are resistant to the widely used anti-estrogen tamoxifen, and broussoflavonol B acts as a potent inhibitor of these breast cancer stem-like cells.

Broussoflavonol B induces differentiation of breast cancer stem-like cells. Based on the cancer stem cell model, tumors originate from transformed stem cells that are able to self-renew and give rise to relatively differentiated cells (cancer progenitor cells) through asymmetric division, thereby forming heterogeneous cell populations found in a tumor (21). Thus, induction of cancer stem cell differentiation or de-stemming of cancer stem cells provides a novel theapeutic option to eliminate cancer stem cells. To examine whether broussoflavonol B also induces differentiation of breast cancer stem cells, we treated SK-BR-3 cells cultured in stem

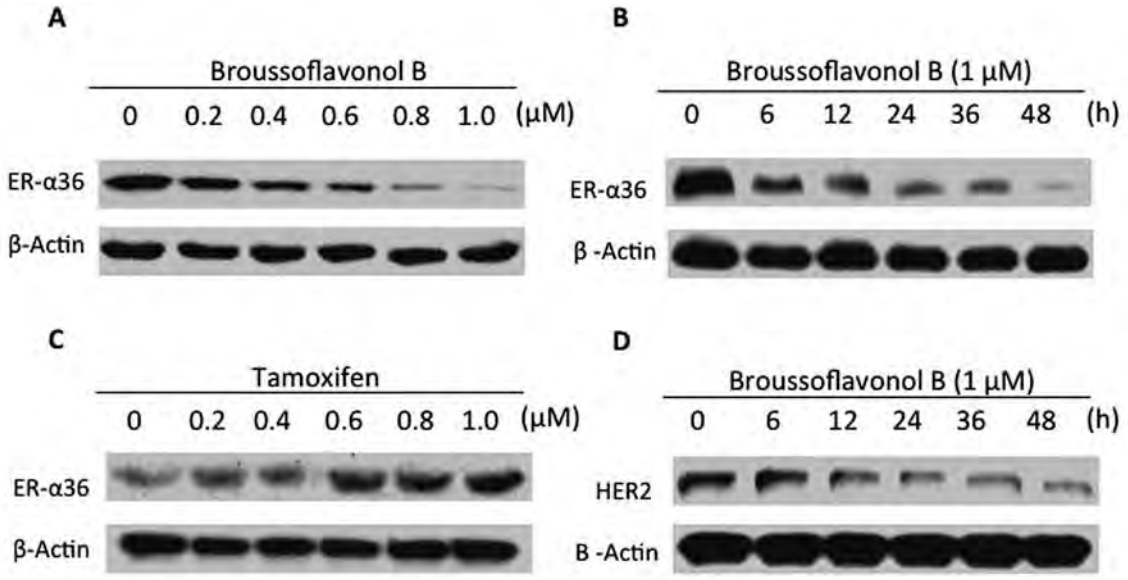

Figure 2. *Broussoflavonol B treatment down-regulates ER-α36 and human epidermal growth factor receptor-2 (HER2) expression. SK-BR-3 cells maintained in phenol red-free medium with 2.5% charcoal-stripped fetal calf serum were treated with dimethyl sulfoxide (DMSO, 0), and the indicated concentrations of broussoflavonol B for 12 h (A) or 1 μM of broussoflavonol B for the indicated hours (h) (B). Cell lysates were then subjected to western blot analysis with an antibody for ER-α36. C. Cells were treated with dimethyl sulfoxide (DMSO, 0) and the indicated concentrations of tamoxifen. Cell lysates were then subjected to western blot analysis with an antibody for ER-α36. D. Cells were treated with DMSO (0) and the indicated concentrations of broussoflavonol B. Cell lysates were then subjected to western blot analysis with an antibody for HER2. All membranes were stripped and re-probed with β-Actin antibody to ensure equal loading.*

cell culture medium with broussoflavonol B and tamoxifen for three days. The cells were then examined for expression of different differentiation markers including CK18 for luminal epithelial differentiation and CD10 for myoepithelial cell differentiation. We found that broussoflavonol B treatment significantly increased the number of cells positive for CK18 and modestly, but significantly increased the number of cells positive for CD10 (Figure 4), suggesting that broussoflavonol B is able to induce differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage and differentiation induction may be one of the mechanisms by which broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells. On the other hand, however, anti-estrogen tamoxifen had less or no effect on the differentiation of these cells (Figure 4), consistent with the previous hypothesis that cancer stem/progenitor cells are resistant to most cancer therapies (9-13).

Discussion

Anti-estrogen tamoxifen has been widely used to treat patients with ER-positive breast tumors, either as adjuvant therapy following surgery, or as first-line treatment for advanced disease. Tamoxifen was also approved as a chemopreventive agent for high-risk women who have a familial history of breast cancer. Although tamoxifen is effective as an adjuvant and chemopreventive agent for ER-positive breast cancer, the therapeutic efficacy of tamoxifen is dramatically reduced in ER-negative tumors. Thus, novel therapeutic agents are urgently needed for treatment of ER-negative breast cancer.

In this study, we investigated the growth-inhibitory potential of a flavonoid derivative broussoflavonol B from the Paper Mulberry tree that grows naturally in Asian and Pacific countries. Crude extracts from this plant exhibit various activities such as anti-platelet activity, inhibition of aromatizing enzymes, anti-oxidant, anti-microbial, anti-inflammatory, inhibition of PTP1B and cytotoxicity. Here, we demonstrated, to our knowledge, for the first time that broussoflavonol B potently inhibited growth of ER-negative SK-BR-3 breast cancer cells, presumably through down-regulation of ER-α36 and HER2 expression. We also demonstrated that broussoflavonol B induced differentiation of breast cancer stem-like cells and restricted the population of ALDH1-positive SK-BR-3 cells.

ER-α36 is highly expressed in ~40% of ER-negative breast cancer cases and its expression is significantly correlated with HER2 expression (18). Recently, we reported the existence of a positive feedback loop between HER2 and ER-α36 expression in SK-BR-3 cells; HER2 signaling activates the promoter activity of ER-α36 and ER-α36 signaling induces *HER2* promoter (19). In addition, we also found that ER-α36

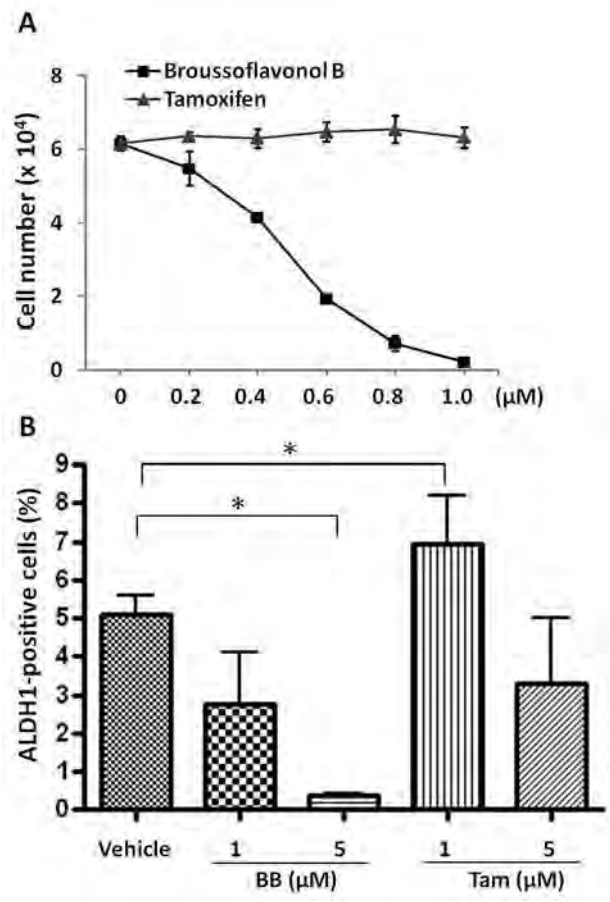

Figure 3. *Broussoflavonol B inhibits growth of ER-negative breast cancer stem-like cells. A: SK-BR-3 cells were maintained in stem cell culture medium and ultra-low attachment dishes and treated with dimethyl sulfoxide (DMSO, 0), the indicated concentrations of broussoflavonol B or tamoxifen for seven days. Cells were then trypsinized and counted. Three dishes were used for each concentration in all of the experiments and all experiments were repeated at least three times. Each point represents the mean±S.D. of three independent experiments. B: SK-BR-3 cells under regular culture conditions were treated with DMSO (0), or the indicated concentrations of broussoflavonol B (BB) or tamoxifen (Tam) for seven days. Cells were then trypsinized and analyzed with flow cytometry after staining with the fluorescent ALDEFLUOR kit. All experiments were repeated three times. Each column represents the mean±S.D. of three independent experiments. *p<0.05.*

is critical for maintenance of ALDH1-positive cancer stem-like SK-BR-3 cells (19), suggesting that down-regulation of ER-α36 expression may provide a novel therapeutic approach to treat ER-negative human breast cancer.

Recently, we reported that the selective estrogen receptor down-regulator (SERD)-ICI 182, 780 effectively down-regulated the expression of ER-α66 protein but increased the steady-state levels of the ER-α36 protein (22), suggesting that ICI 182, 780 only disrupts ER-α66 protein. Here, we found that broussoflavonol B potently down-regulated ER-α36 expression at sub-micromolar concentrations while tamoxifen had no effect, suggesting that broussoflavonol B is an ER-α36 down-regulator that may inhibit the non-genomic estrogen signaling mediated by ER-α36. In addition, we also found that broussoflavonol B down-regulated the steady-state levels of HER2 protein. Thus, our results strongly suggest that disruption of the positive feedback loop between HER2 and ER-α36 attenuates mitogenic signaling and restricts the malignant growth of ER-negative SK-BR-3 cells.

Accumulating evidence indicates that many types of cancer, including breast cancer, originate from and are maintained by a small population of cancer stem/progenitor cells (23). In this study, we showed that ER-negative breast cancer stem-like cells were also resistant to the anti-estrogen tamoxifen, consistent with the previous reports that cancer stem/progenitor cells are resistant to many current cancer therapies including chemo- and radiation therapy (9-13). However, broussoflavonol B inhibited growth of ALDH1-positive breast cancer stem-like cells and induced their differentiation.

According to the cancer stem cell model, tumors originate from cancer stem cells that are able to differentiate into non-cancer cells (1, 3). Thus, it was postulated that induction of cancer stem cell terminal differentiation or de-stemming of cancer stem cells may provide with a novel theapeutic option to eliminate cancer stem cells (24). Recently, it was shown that bone morphogenic protein-4 enhanced terminal differentiation, apoptosis and chemosensitization of colorectal cancer stem cells (25), suggesting the possibility of ligand-induced differentiation therapy. Here, we demonstrated that broussoflavonol B potently induced differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage. We also found that broussoflavonol B was able to inhibit the growth of these stem-like cells, suggesting that broussoflavonol B may induce terminal differentiation of ER-negative breast cancer stem-like cells. Our results strongly indicate that induction of cancer stem-like cell terminal differentiation is a feasible theapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

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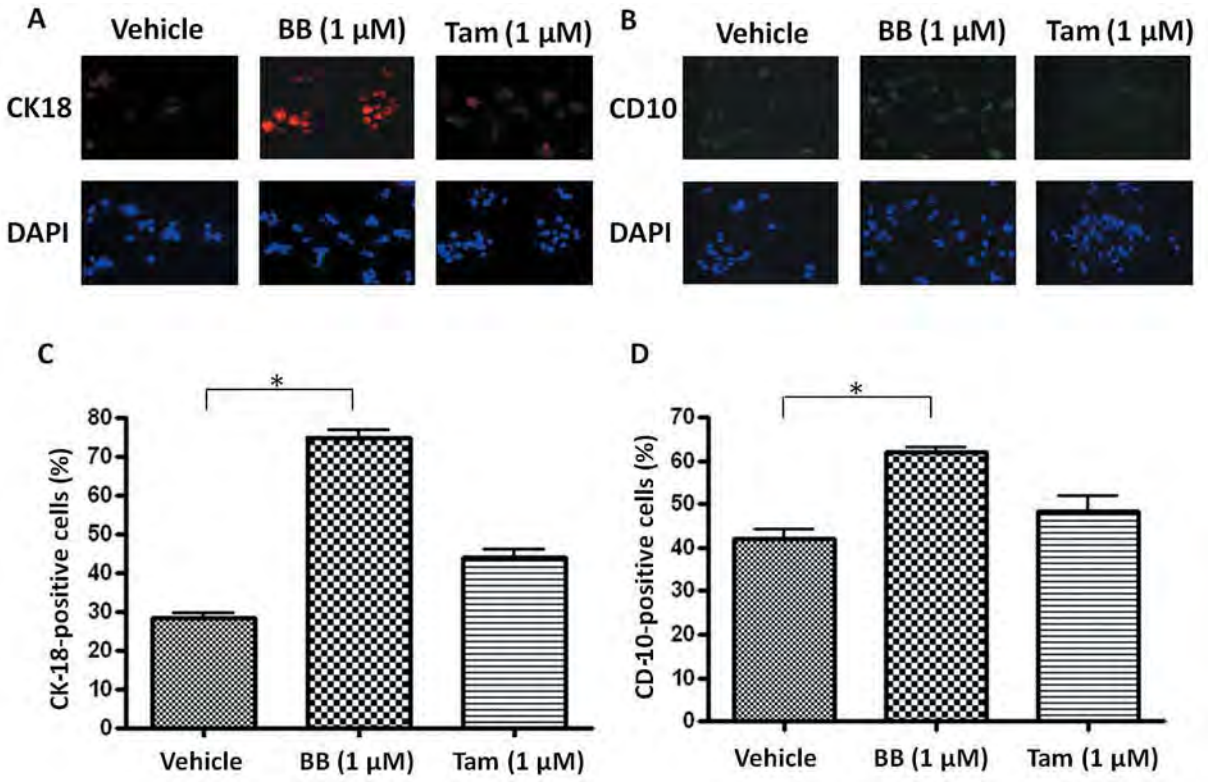

Figure 4. *Broussoflavonol B induces differentiation of ER-negative breast cancer stem-like cells. SK-BR-3 cells maintained in stem cells culture medium and ultra-low attachment dishes were treated with dimethyl sulfoxide (DMSO, vehicle), or 1 μM of broussoflavonol B (BB) or tamoxifen (Tam) for three days. Cells were then cytospinned onto slides, stained with antibodies against CK18 or CD10 and appropriate secondary antibodies, and photographed using a Zeiss fluorescence microscope. Five hundred cells were then assessed for CD10 and CK18 positivity under a fluorescence microscope and the percentage of cells positive for these markers was calculated. The experiment was repeated three times. Each column represents the mean±S.D. of three independent experiments. *p<0.01.*

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Endocrine pharmacology

A novel anticancer agent Broussoflavonol B downregulates estrogen receptor (ER)-α36 expression and inhibits growth of ER-negative breast cancer MDA-MB-231 cells

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ABSTRACT

Estrogen receptor (ER)-negative breast cancers are aggressive and unresponsive to antiestrogens, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapies are urgently needed to treat this type of breast cancer. Here, we report that Broussoflavonol B, a chemical purified from the bark of the Paper Mulberry tree (*Broussonetia papyrifera*) exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar concentrations. Broussoflavonol B induced cell cycle arrest at both the G_0/G_1 and G_2/M phases accompanied by a downregulation of c-Myc protein, a upregulation of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ and a down-regulation of the expression levels of the G_2/M regulatory proteins such as cyclin B1, cdc2 and cdc25C. Broussoflavonol B also induced apoptotic cell death characterized by accumulation of the annexin V- and propidium iodide-positive cells, and cleavage of caspases 8, 9 and 3. In addition, Broussoflavonol B treatment also decreased the steady state levels of the epidermal growth factor receptor (EGFR) and ER-α36, a variant of estrogen receptor-α, and restricted growth of the stem-like cells in ER-negative breast cancer MDA-MB-231 cells. Our results thus indicate that Broussoflavonol B is a potent growth inhibitor for ER-negative breast cancer cells and provide a rational for preclinical and clinical evaluation of Broussoflavonol B for ER-negative breast cancer therapy.

1. Introduction

Depending on the existence of one of the estrogen receptors, ER-α, human breast cancers are divided into ER-positive or ER-negative. Approximately 70% of breast cancer patients are positive for ER-α and these patients are suitable for hormonal therapy that blocks estrogen stimulation of breast cancer cells. However, ER-negative breast cancer that accounts for about one third of breast cancers diagnosed is often more malignant and aggressive than ER-positive breast cancer. In addition, ER-negative breast cancer patients respond poorly to antiestrogen therapy, and current therapeutic modalities for ER-negative breast cancer patients are usually associated with strong toxicity and side effects. Less toxic and more effective targeted therapeutic approaches are urgently needed to treat this type of breast cancer.

Previously, we identified and cloned a 36 kDa variant of ER-α, ER-α36, that is mainly expressed outside of the cell nucleus and mediates non-genomic estrogen signaling (Wang et al., 2005, 2006). ER-α36 lacks both transcription activation function domains AF-1 and AF-2 of the full-length 66 kDa ER-α (ER-α66), consistent with the fact that ER-α36 has no intrinsic transcriptional activity (Wang et al., 2006). ER-α36 is generated from a promoter located in the first intron of the ER-α66 gene (Zou et al., 2009), indicating that ER-α36 expression is regulated differently from ER-α66, consistent with the findings that ER-α36 is expressed in specimens from ER-negative breast cancer patients and established ER-negative breast cancer cells that lack ER-α66 expression (Pelekanou et al., 2012; Shi et al., 2009; Vranic et al., 2011; Wang et al., 2006). ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011); ER-negative breast cancer MDA-MB-231 and -436 cells with knocked-down concentrations of ER-α36 protein failed to form xenograft tumors in nude mice. Thus, ER-α36-mediated signaling plays an important role in development and progression of ER-negative breast cancer, and ER-α36 may be used as a target to develop novel and more

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effective therapeutic agents for treatment of ER-negative breast cancer.

Broussonetia papyrifera (Moraceae), also known as paper mulberry, grows naturally in Asia and Pacific countries. Its dried fruits have been used for the treatment of ophthalmic disorders and impotency (Lee et al., 2001). The leaves, twig roots and barks of this plant are widely used to treat gynecological bleeding, dropsy, dysentery diseases as a folk medicine in China (Feng et al., 2008). Various types of flavonoids are the major constituents of this plant and some of which exhibited strong tyrosinase inhibitory (Zheng et al., 2008), aromatase inhibitorory (Lee et al., 2001), antifungal (Takasugi et al., 1980, 1984), secretory phospholipase A-2 inhibitory (Kwak et al., 2003), PTP1B enzyme inhibitory (Chen et al., 2002; Nguyen et al., 2012), antimicrobial, cytotoxic (Sohn et al., 2004), antiplatelet (Lin et al., 1996), antioxidant and inducible nitric oxide synthase suppressing activities (Cheng et al., 2001). However, the effects and the underlying mechanisms of the flavonoids from *B. papyrifera* in human cancer have never been studied. Recently, we purified and identified two prenylflavone derivatives from *B. papyrifera* (Guo et al., 2013); one is a known compound Broussonol D (Zhang et al., 2011) that has been isolated from Broussonetia kazinoki and another is Broussoflavonol B (Matsumoto et al., 1985). We also found that both compounds were able to inhibit growth of ER-positive breast cancer MCF7 cells presumably through down-regulation of ER-α36 expression (Guo et al., 2013). However, Broussoflavonol B was more potent than Broussonol D in downregulation of ER-α36 expression (Guo et al., 2013). Since ER-α36 is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011), we decided to study the effects and underlying mechanisms of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) purified from the bark of *B. papyrifera* in growth of ER-negative breast cancer MDA-MB-231 cell.

In the present study, we demonstrated that Broussoflavonol B exhibited potent growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells at sub-micromolar (μM) concentrations. Broussoflavonol B treatment decreased the steady state levels of ER-α36 and EGFR proteins, and induced cell cycle arrest and cell apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

Broussoflavonol B (99.5% purity) was obtained from Shenogen Parma Group, Ltd (Beijing, China). Anti-p16^{INK4a} (N-20), p19^{INK4D} (M-167), p21$^{WAF1/CIP1}$ (F-5), β-actin (I-19), c-Myc (9E10), caspase 3 (S-19), caspase 8 (H-134), caspase 9 (H-170), Cdc 25c (H-150), cyclin B1 (GNS1) and Cdc2 p34 (POH-1), cytokeratin 18 (DC-10), CD10 (H-321) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-EGFR antibody (1F4) and anti-vimentin (D21H3) antibody were from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-ER-α36 antibody was generated and characterized as described before (Wang et al., 2006).

2.2. Cell culture

MDA-MB-231 cells were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution from Invitrogen Life Technologies Corporation (Carlsbad, CA, USA). Before experiments, cells were maintained in phenol red-free media with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA).

2.3. Cell growth and differentiation assays

Cells in the phenol red-free medium were seeded onto 35 mm dishes at 5×10^4 cells/dish. After 24 h, the indicated concentrations of vehicle dimethyl sulfoxide (DMSO), Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were trypsinized and counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each concentration point and experiments were repeated at least three times.

To assess the effects of Broussoflavonol B on epidermal growth factor (EGF)-stimulated cell growth, cells (1×10^4/dish) in 60 mm dishes were maintained in phenol red-free medium with 2.5% charcoal-stripped FBS (Thermo Scientific Hyclone, Logan, UT, USA) for 48 h. EGF (10 ng/ml) alone, together with Broussoflavonol B or Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea). Three dishes were used for each treatment and experiments were repeated more than three times.

For cancer stem-like cell growth, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate (Corning Incorporated, CA, USA) at 10,000 cells/ml and cultured in phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 1X B27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). The different concentrations of Broussoflavonol B or tamoxifen were

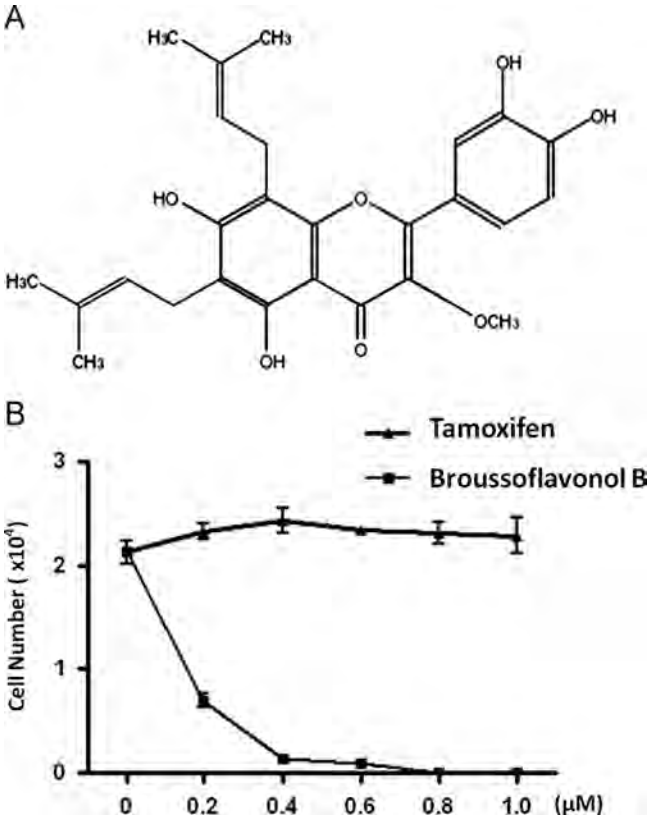

Fig. 1. Broussoflavonol B inhibits growth of ER-negative breast cancer MDA-MB-231 cells. (A) The chemical structure of Broussoflavonol B (5,7,3′,4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone). (B) Effects of Broussoflavonol B or tamoxifen on the growth of MDA-MB-231 cells. Cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with vehicle, DMSO (0) and 0.2, 0.4, 0.6, 0.8 and 1.0 μM of Broussoflavonol B or tamoxifen for seven days before cells were trypsinized and counted. Three dishes were used for each concentration in the experiments and all experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments.

added and incubated for seven days. Cells were collected, washed with PBS, and incubated with Trypsin-EDTA (0.05%/0.5 mM) for 2 min at 37 °C, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

For the differentiation assay of cancer stem-like cells, MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 10 cm dishes (Corning Incorporated) at 10,000 cells/ml and cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected, washed with phosphate buffered saline (PBS) and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CD10, vimentin or CK18 antibodies. Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope (Nikon, Eclipss E600), and the percentage of cells positive for these markers were calculated.

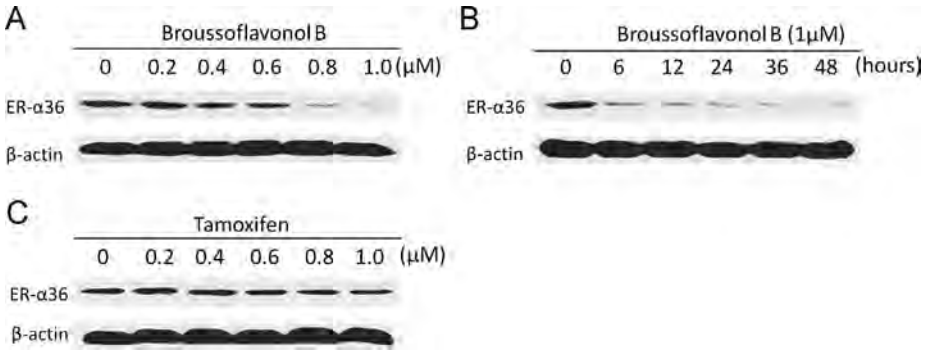

Fig. 2. Broussoflavonol B treatment downregulates ER-α36 expression. ((A) and (B)) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h or 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for ER-α36. (C) Cells were treated with DMSO (0) and indicated concentrations of tamoxifen. Cell lysates were subjected to Western blot analysis with the antibody for ER-α36. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

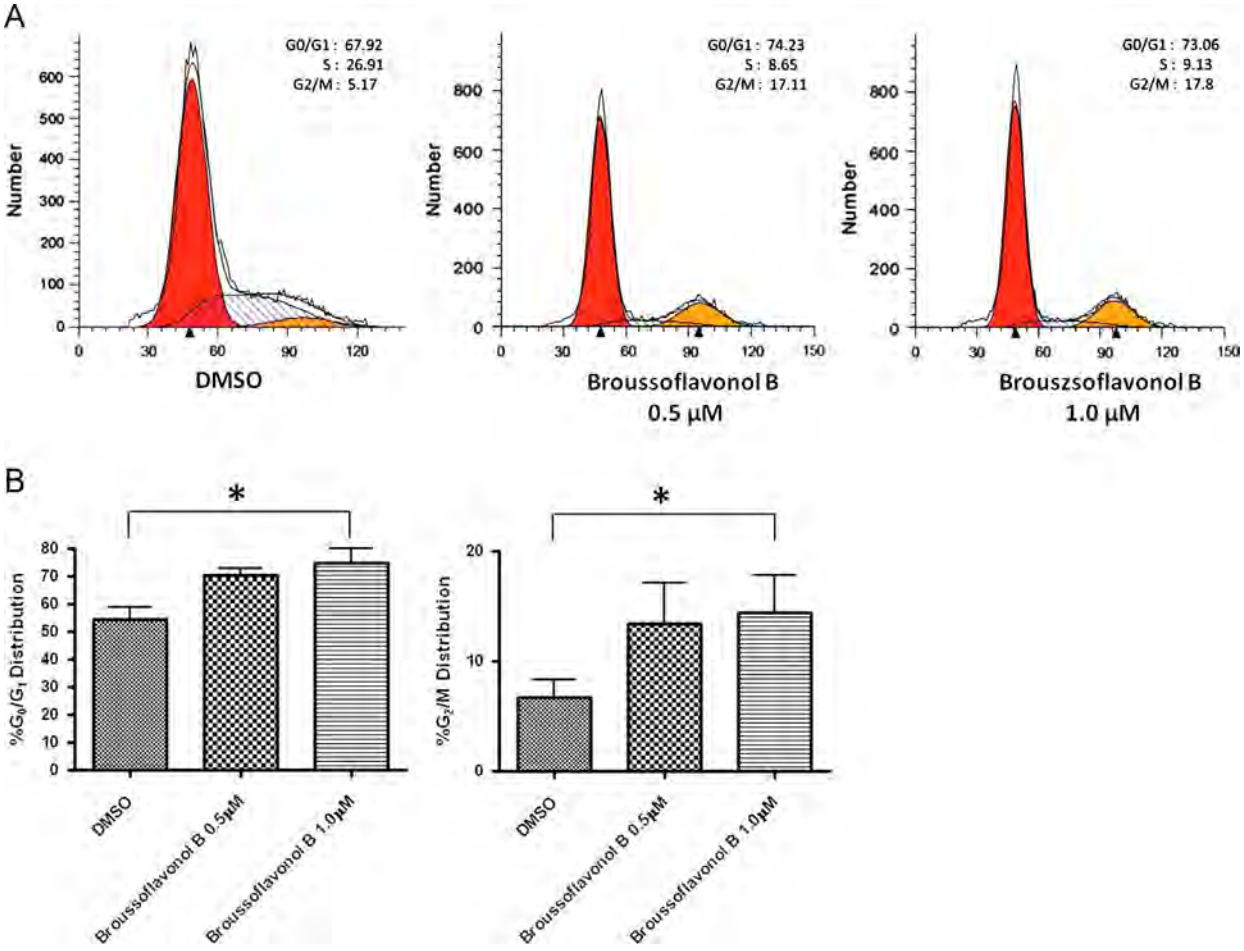

Fig. 3. Broussoflavonol B induces G_0/G_1 and G_2/M arrest of the cell cycle in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with DMSO (vehicle) or the indicated concentrations of Broussoflavonol B for 72 h. Cells were assayed with PI staining and flow cytometric analysis. All experiments were repeated three times and data from a representative experiment is shown. (B) Percentage of cells in the G_0/G_1 and G_2/M phases of the cell cycle in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. * $P < 0.05$.

2.4. Western blot assay

Cells were washed with cold PBS twice and lysed with the RIPA buffer containing 1% proteinase inhibitor and 1% phosphatase inhibitor cocktail solution (Sigma, St. Louis, MO, USA). The cell lysates were boiled for 5 min in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.5. Cell cycle and cell death analysis

Cells at ~70% confluence were harvested and 1 ml of cold 70% ethanol was slowly added to the cell pellet while vortexing. Ethanol-fixed cells were treated with 100 μg/ml RNaseA and 50 μg/ml propidium iodide (PI) in PBS at room temperature for 30 min. Flow cytometry analysis of cell cycle distribution was performed using a FACSCalibur flow cytometer (BD-Biosciences).

Cell death was detected using the annexin V-FITC apoptosis kit (Invitrogen) according to the manufacturer's instruction. Data acquisition was performed with the CellQuest software and analyzed with the ModFit software.

2.6. Statistical analysis

Data were summarized as the means ± standard deviation (S.D.) using GraphPad InStat software program. Statistical analysis was performed using paired-samples *t*-test, or ANOVA followed by the Student–Newman–Keuls testing and the significance was accepted for *P* values less than 0.05.

3. Results

3.1. Broussoflavonol B exhibits growth inhibitory activity in ER-negative breast cancer MDA-MB-231 cells

Previously, we reported that ER-α36 plays an critical role in malignant growth of ER-negative breast cancer MDA-MB-231 cells (Zhang et al., 2011), suggesting the downregulation of ER-α36 expression may provide a novel approach to inhibit growth of ER-negative breast cancer cells. Recently, we found that a flavonoid, Broussoflavonol B (5, 7, 3′, 4′-Tetrahydroxy-3-methoxy-6,8-diprenylflavone) (Fig. 1A) purified from the bark of *B. papyrifera* was able to downregulate ER-α36 expression and inhibit proliferation in ER-positive breast cancer MCF7 cells (Guo et al., 2013).

To examine the effects of this chemical on growth of ER-negative breast cancer cells, we used ER-negative breast cancer MDA-MB-231 cells as a model to perform cell growth inhibition assay. Cells were incubated with increasing concentrations of Broussoflavonol B or the classical anti-estrogen tamoxifen for seven days, and the cell numbers were then counted. Broussoflavonol B potently inhibited growth of MDA-MB-231 cells at sub-μM concentrations while the classical anti-estrogen tamoxifen had no effect (Fig. 1B). Our result thus suggested that Broussoflavonol B exhibited growth inhibitory activity in ER-negative breast cancer cells.

3.2. Broussoflavonol B downregulates ER-α36 expression in MDA-MB-231 cells

To probe the molecular mechanisms by which Broussoflavonol B inhibited growth of MDA-MB-231 cells, we assessed the effects of Broussoflavonol B on expression of ER-α36, a protein important for malignant growth of MDA-MB-231 cells (Zhang et al., 2011). Western blot analysis indicated that Broussoflavonol B treatment downregulated ER-α36 expression in a dose- and time-dependent manner (Fig. 2A and B) whereas the classic anti-estrogen tamoxifen was without any effect on ER-α36 expression (Fig. 2C). Thus, our data suggested that ER-α36 downregulation is a mechanism underlying Broussoflavonol B growth inhibitory activity in these cells.

3.3. Broussoflavonol B induces both the G_0/G_1 and G_2/M phase arrest in MDA-MB-231 cells

To further examine the mechanisms underlying Broussoflavonol B growth inhibitory activity, we also studied its effect on the cell cycle progression. Cell populations in the G_0/G_1, S and G_2/M phases of the cell cycle were determined with propidium iodide (PI) staining followed by flow cytometry. Broussoflavonol B treatment increased the population of MDA-MB-231 cells in both the G_0/G_1 and G_2/M phases accompanied with a dramatically reduced population of the S phase; for vehicle (DMSO), 0.5 and 1 μM Broussoflavonol B, respectively (Fig. 3A and B).

We also examined the effects of Broussoflavonol B on the expression of the proteins involved in regulation of the G_1/S transition of the cell cycle including c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Western blot analysis showed that Broussoflavonol B treatment down-regulated the expression levels of the growth promoting protein c-Myc in a dose-dependent manner (Fig. 4A) while induced expression levels of the cell cycle inhibitory proteins $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$ (Fig. 4A).

We then examined the effect of Broussoflavonol B on the expression of the proteins critical for the G_2/M transition including cyclin B1, cdc2 and cdc25C. Western blot analysis showed that

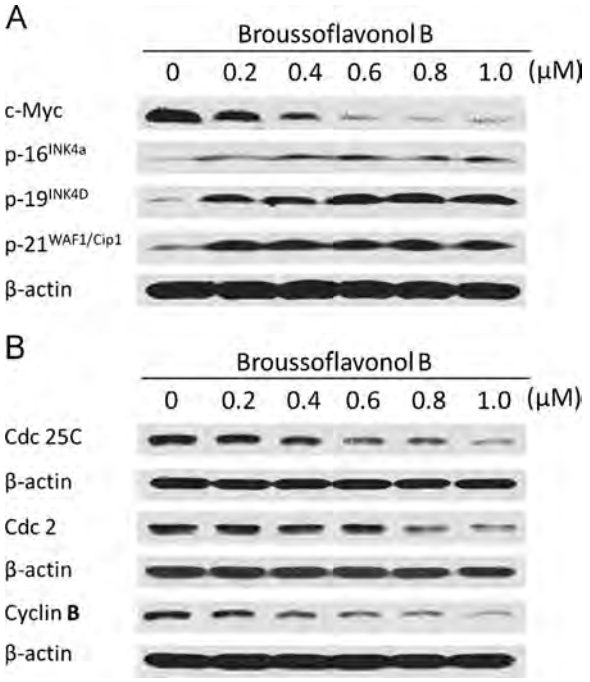

Fig. 4. Broussoflavonol B regulates expression levels of the cell cycle regulators in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for c-Myc, $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. (B) MDA-MB-231 **c**ells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for cyclin B1, cdc2 and cdc25C. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

Broussoflavonol B treatment down-regulated the expression levels of cyclin B1, cdc25C and cdc2 in a dose-dependent manner (Fig. 4B). Altogether, these results demonstrated that Broussoflavonol B treatment arrested ER-negative breast cancer MDA-MB-231 cells at both the G_0/G_1 and G_2/M phases of the cell cycle.

3.4. Broussoflavonol B activates caspase-mediated cell apoptosis in MDA-MB-231 cells

During our experiments, we also noticed that there were floating cells in the MDA-MB-231 cells treated with Broussoflavonol B. We decided to determine whether Broussoflavonol B also induces cell apoptosis. MDA-MB-231 cells were treated with different concentrations of Broussoflavonol B for 48 h, and the annexin V-FITC and propidium iodide (PI) fluorescence assays were performed to examine the early stage apoptotic cells (annexin-positive/PI-negative), the late stage apoptotic cells (annexin-positive/PI-positive), and necrotic cells (annexin-positive/PI-positive). In MDA-MB-231 cells treated with 0.5 and 1 μM of Broussoflavonol B, cells were induced to apoptotic and/or necrotic cell death as shown as increased cell populations in groups of annexin V-positive/ PI-negative, annexin V-positive/PI-positive and annex V-negative/PI-positive (Fig. 5A and B).

We also examined whether the caspase cascades were activated in Broussoflavonol B treated cells. Western blot analysis revealed that Broussoflavonol B treatment resulted in a dose-dependent activation of the initiator caspases 8, 9 and the executor caspase 3. As shown in Fig. 5C, the levels of remaining procaspases 8, 9 and 3 in Broussoflavonol B treated cells were dramatically decreased compare to the control cells treated with DMSO vehicle. Taken together, our results demonstrated that Broussoflavonol B induces apoptotic as well as necrotic cell death in ER-negative breast cancer MDA-MB-231 cells.

3.5. Broussoflavonol B downregulates EGFR expression and inhibits EGF-stimulated growth of MDA-MB-231 cells

Recently, we reported that ER-α36 positively regulates the stability of the epidermal growth factor receptor (EGFR) protein; knockdown of ER-α36 expression destabilized EGFR protein (Zhang et al., 2011). We decided to examine whether down-regulated ER-α36 expression by Broussoflavonol B also down-regulates EGFR expression. We thus examined EGFR expression in MDA-MB-231 cells treated with Broussoflavonol B, and found that Broussoflavonol B also decreased the steady state levels of EGFR protein (Fig. 6A). MDA-MB-231 cells represent a typical triple-negative breast cancer that lacks expression of estrogen receptor, progesterone receptor and HER2, and often relies on EGFR signaling for malignant growth. We then decided to examine whether Broussoflavonol B is able to inhibit EGF-stimulated cell growth in MDA-MB-231 cells. In serum-starved cells, addition of

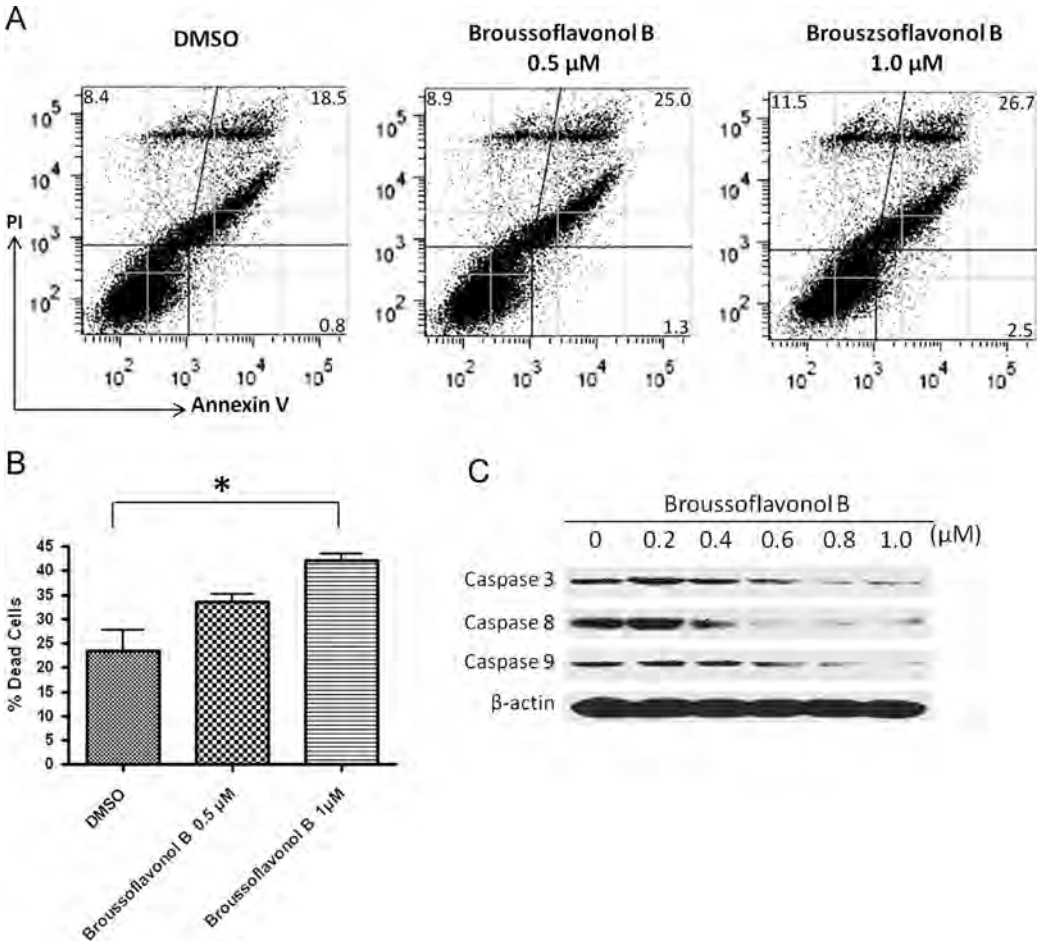

Fig. 5. Broussoflavonol B induces cell death in MDA-MB-231 cells. (A) MDA-MB-231 cells maintained in phenol red-free media with 2.5% charcoal-stripped fetal calf serum were treated with DMSO (0) or 0.5 and 1 μM Broussoflavonol B for 48 h. Cells were collected and analyzed with flow cytometric analysis after annexin V and PI staining. The experiment was repeated three times and the results from a representative experiment were shown. (B) Percentage of dead cells in MDA-MB-231 cells treated with or without Broussoflavonol B. Each column represents mean ± S.D. of three independent experiments. * $P < 0.05$. (C) MDA-MB-231 cells were treated with DMSO (0), and indicated concentrations of Broussoflavonol B for 12 h. Cell lysates were subjected to Western blot analysis with antibodies for caspases 3, 8 and 9. All membranes were stripped and re-probed with a β-actin antibody to ensure equal loading.

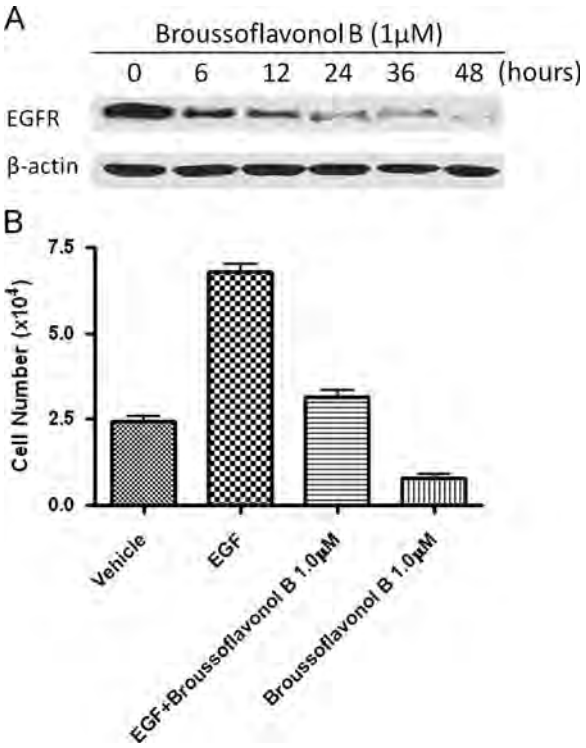

Fig. 6. Broussoflavonol B downregulates EGFR expression and attenuates mitogenic EGF signaling in MDA-MB-231 cells. (A) MDA-MB-231 **c**ells were treated with 1 μM of Broussoflavonol B for indicated time periods. Cell lysates were subjected to Western blot analysis with an antibody for EGFR. The membrane was stripped and re-probed with a β-actin antibody to ensure equal loading. (B) Cells maintained in phenol red-free medium with 2.5% charcoal-stripped FBS for 48 h. EGF (10 ng/ml) alone, together with 1 μM Broussoflavonol B or 1 μM Broussoflavonol B alone were added to cells and incubated for 72 h, and the cell numbers were determined. Three dishes were used for each treatment and experiments were repeated more than three times.

EGF (10 ng/ml) stimulated cell proliferation, which was inhibited by inclusion of Broussoflavonol B, indicating that Broussoflavonol B also attenuates mitogenic EGF signaling presumably via down-regulation of EGFR expression.

3.6. Broussoflavonol B inhibits the growth of breast cancer stem-like cells in MDA-MB-231 cells

Recently, we reported that ER-α36 is important in maintenance of the stem-like cells in ER-negative breast cancer SK-BR-3 cells (Kang et al., 2011). We decided to test the inhibitory effects of Broussoflavonol B on the stem-like cells in MDA-MB-231 cells. To this aim, we cultured MDA-MB-231 cells in a stem cell medium using ultralow-attachment dishes, which enriched the breast cancer stem-like cells. These stem-like cells were than treated with the increasing concentrations of tamoxifen or Broussoflavonol B for seven days. We found that Broussoflavonol B effectively inhibited the growth of these stem-like breast cancer cells while tamoxifen had less effect (Fig. 7A).

To examine whether Broussoflavonol B induces differentiation of breast cancer stem-like cells, we treated MDA-MB-231 cells cultured in the stem cell medium with indicated concentrations of Broussoflavonol B for three days. The cells were then examined for expression of differentiation markers including cytokeratin 18 (CK18) for luminal epithelial differentiation, CD10 for myoepithelial cell differentiation and vimentin for mecenchymal cell differentiation. We found that Broussoflavonol B treatment significantly increased the number of cells positive for CK18 while had no effect on the number of cells positive for vimentin (Fig. 7B). We did not observe CD10 staining in Broussoflavonol B treated cells (data not shown). Our results indicated that Broussoflavonol B is able to induce differentiation of ER-negative breast cancer stem-like cells mainly into the luminal epithelial lineage and differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells.

4. Discussion

In this study, we investigated the growth inhibitory potential of a flavonoid derivative Broussoflavonol B from the Paper Mulberry tree (*B. papyrifera*). *B. papyrifera* has been used for cancer, dyspepsia, and pregnancy (Johnson, 1998). In mainland China, the fruits of *B. papyrifera* have been employed for impotency and ophthalmic disorders (Matsuda et al., 1995). Crude extracts or purified compounds from *B. papyrifera* have exhibited various biological activities, such as anti-proliferation, antioxidative, aromatase inhibitory, cytotoxic, glycosidase inhibitory, and platelet aggregation inhibitory effects (Lee and Kinghorn, 2003).

Currently, seven broussoflavonols were purified from *B. papyrifera* and named as broussoflavonols A through G (Lee and Kinghorn 2003). Broussoflavonols E and F exhibited platelet aggregation inhibitory activity partially due to an inhibitory effect on cyclooxygenase (Lin et al., 1996). Both Broussoflavonols F and G potently inhibited Fe^{2+}-induced lipid oxidation in rat-brain homogenate and significantly inhibited the proliferation of rat vascular smooth muscle cells (Ko et al., 1997). Recently, we reported that Broussoflavonol B significantly inhibited growth of ER-positive breast cancer cells (Guo et al., 2013). Here, we demonstrated that Broussoflavonol B also potently inhibited growth of triple-negative and basal-like breast cancer MDA-MB-231 cells through down-regulation of ER-α36 and EGFR expression and induction of the G_0/G_1 and G_2/M arrest of the cell cycle as well as cell death. We also showed that Broussoflavonol B reduced the population of breast cancer stem-like cells.

The ER-α variant, ER-α36, is highly expressed in ~40% of ER-negative breast cancer (Shi et al., 2009) and its expression is significantly correlated with expression of members of the EGFR family such as EGFR and HER2 (Shi et al., 2009; Zhang et al., 2011). Recently, we reported the existence of a positive feedback loop between EGFR and ER-α36 expression in ER-negative breast cancer cells, which is critical for malignant growth of ER-negative breast cancer cells (Zhang et al., 2011). Here, we reported that Broussoflavonol B potently downregulated ER-α36 expression at sub-μM while antiestrogen tamoxifen was without any effects. In addition, Broussoflavonol B also downregulated the levels of EGFR protein since ER-α36 protein is important for stability of EGFR protein (Zhang et al., 2011), and attenuated the mitogenic EGF signaling that is critical for malignant growth of MDA-MB-231 cells. Thus, our results suggested that disruption of the positive regulatory loop between ER-α36 and EGFR through down-regulation of ER-α36 provides an effective approach to inhibit growth of ER-negative breast cancer cells.

Eukaryotic cell cycle progression involves sequential activation of Cdks, which are controlled by a complex of proteins, including the cyclins. Here, we found that Broussoflavonol B treatment also arrested MDA-MB-231 cells mainly at the G_2/M phase of the cell cycle, which was accompanied with down-regulation of the expression levels of the proteins pivotal for the G_2/M transition. We also found that Broussoflavonol B modestly arrested the cell cycle at the G_0/G_1 phase. Cell-cycle progression involves sequential activation of cyclins and cyclin-dependent kinases (CDKs). To prevent abnormal proliferation, cyclin-CDK complexes are negatively regulated by cell cycle inhibitors (Sherr and Roberts, 1999). Here, we found that in Broussoflavonol B treated MDA-MB-231

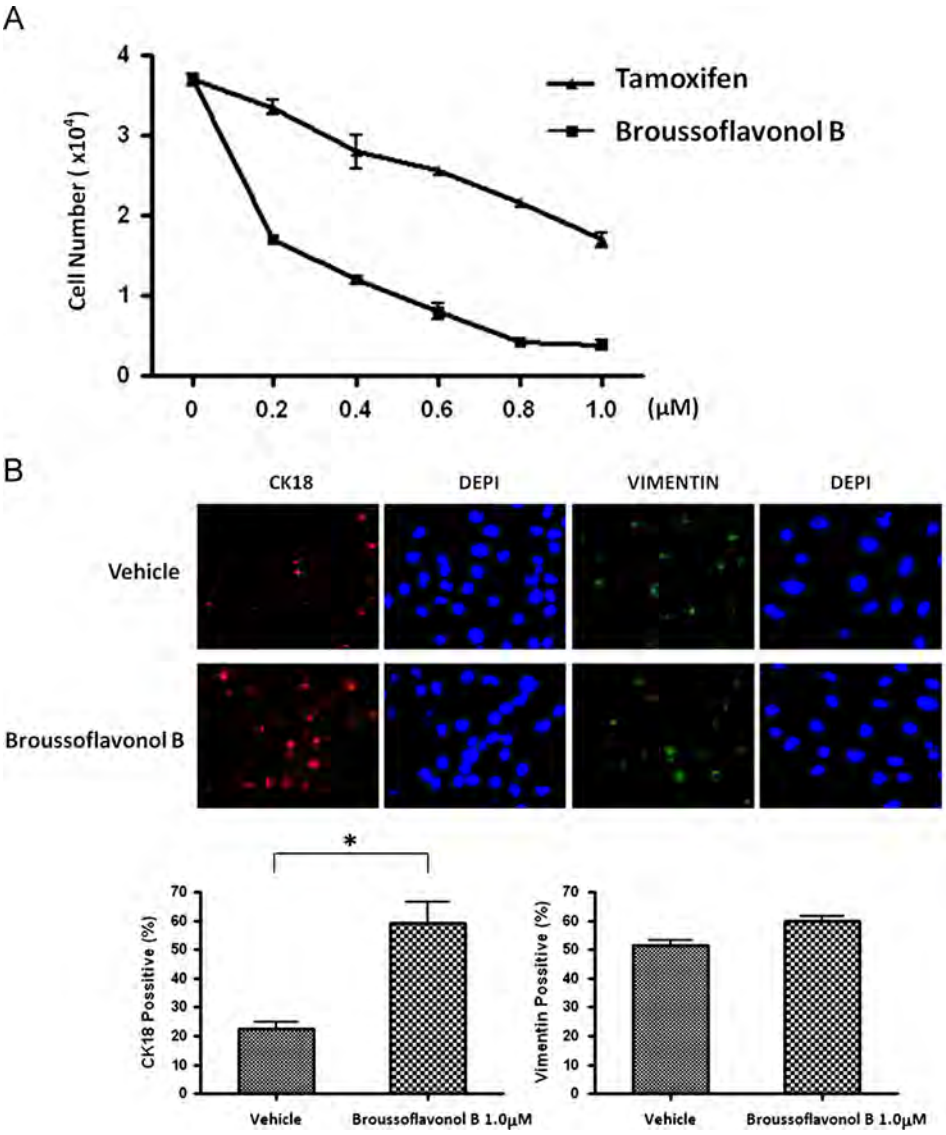

Fig. 7. Broussoflavonol B inhibits growth of ER-negative breast cancer stem/progenitor cells. (A) MDA-MB-231 cells were seeded onto Corning Ultra-Low Attachment 6-well plate and cultured in stem cell medium. Different concentrations of Broussoflavonol B or tamoxifen were added and incubated for seven days. Cells were counted using the ADAM automatic cell counter. Three dishes were used for each treatment and experiments were repeated three times. Each point represents mean ± S.D. of three independent experiments. (B) MDA-MB-231 cells were cultured in the stem cell medium for seven days. Indicated concentrations of Broussoflavonol B were added and incubated for another three days. Cells were collected and cytospined onto slides. Cytospined slides were stained with indirect immunofluorescent staining using anti-CK18 and vimentin antibodies (upper panels). Five hundred cells were assessed for vimentin or CK18 positivity under the fluorescent microscope and the percentage of cells positive for these markers were calculated (lower panels). The experiments were repeated three times. $^{*}P < 0.01$.

cells, the G_0/G_1 arrest of the cell cycle was accompanied with down-regulation of the growth-promoting protein c-Myc and induction of the cell cycle inhibitors including $p16^{INK4a}$, $p19^{INK4D}$ and $p21^{WAF1/CIP1}$. Thus, The G_0/G_1 phase arrest is also involved in Broussoflavonol B inhibitory function in growth of ER-negative MDA-MB-231 cells.

Furthermore, flow cytometric analysis using Annexin V/PI staining demonstrated that Broussoflavonol B could dose-dependently induced apoptosis in MDA-MB-231 cells. Caspases are the principal effectors of apoptosis involved in pathways such as caspase 8 regulated extrinsic and caspase 9-regulated intrinsic pathways. The caspase 9 pathway links mitochondrial damage to caspase activation, and serves as an index of damage in mitochondrial membrane function (Bao and Shi, 2007). In addition, the downstream member caspase 3 is an executor of DNA fragmentation (Bao and Shi, 2007). As expected, we observed that Broussoflavonol B treatment induced activation of caspases 8, 9 and 3. Thus, proteolytic processing of the initiator caspases as well as the executor caspase, and subsequent apoptosis contributed to growth inhibitory activity of Broussoflavonol B.

Accumulating evidence indicated that many types of cancer, including breast cancer, are initiated from cancer stem/progenitor cells (Liu et al., 2005; Charafe-Jauffret et al., 2009). These cancer stem/progenitor cells are resistant to most therapeutic approaches currently used (Dean, 2006; Diehn and Clarke, 2006; O'Brien et al., 2009; Hambardzumyan et al., 2006; Shafee et al., 2008). In this study, we showed that ER-negative breast cancer stem-like cells were resistant to anti-estrogen tamoxifen, consistent with the concept that cancer stem/progenitor cells are resistant to current cancer therapies. Thus, the development of novel drugs that are able to selectively attack the cancer stem cells is of the greatest priority. Recently a large scale screening was conducted to seek agents selectively kill epithelial cancer stem cells, and salnomycin was identified as a potent agent specifically targeting breast cancer stem cells (Gupta et al., 2009). More recently, dietary chemopreventive agents sulforaphane and benzyl isothiocyanate were

reported to be able to inhibit growth of breast cancer stem cells both in vitro and in vivo (Li et al., 2010; Kim et al., 2013). It is worth noting that we have previously reported that phenethyl isothiocyanate acted more potently than the "pure" antiestrogen ICI 182,780 to down-regulates ER-α36 expression and to inhibit breast cancer cell growth (Kang and Wang, 2010). Together with our current finding, these results suggested that down-regulation of ER-α36 and function is a novel approach to target breast cancer stem/progenitor cells.

In this study, we also found that Broussoflavonol B not only inhibited growth of ER-negative breast cancer stem-like cells but also induced differentiation of these cells, suggesting that differentiation induction may be one of the mechanisms by which Broussoflavonol B restricts growth of ER-negative breast cancer stem-like cells. Based on the cancer stem cell model, tumors are originated from malignantly transformed stem cells that are able to self-renew (Clarke et al., 2006). Thus, induction of cancer stem cell differentiation or "destemming" cancer stem cells provides a novel therapeutic option to eliminate cancer stem cells. Thus, our results demonstrated that induction of cancer stem-like cell terminal differentiation or "destemming" cancer stem cells is a feasible therapeutic approach to eradicate human breast cancer by eliminating cancer stem cells.

5. Conclusion

Our results demonstrated that Broussoflavonol B from the Paper Mulberry tree possesses potent anti-growth activity; inducing the arrest of the cell cycle and cell death in ER-negative breast cancer cells. Broussoflavonol B also effectively downregulates the steady state levels of ER-α36 and EGFR proteins, indicating that Broussoflavonol B acts like a selective estrogen receptor downregulator (SERD) and a disruptor of the positive regulatory loop consisted of ER-α36 and EGFR in ER-negative breast cancer cell. More importantly, our results also indicated that Broussoflavonol B restricts growth of breast cancer stem-like cells. Thus, our results provide experimental evidence for the hypothesis that ER-α36 can serve as a target to develop novel and effective therapeutic approaches for ER-negative breast cancer.

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